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Author Index

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A brief (200 word maximum) Abstract should be provided. The use in the Abstract of numbers to identify compounds should be avoided unless these compounds are also identified by names.

A list of three to five keywords must be given and placed after the Abstract. Keywords may be single words or very short phrases.

Although variations in accord with contents of a manuscript are permissible, in general all papers should have the following sections: Introduction, Materials and Methods, Results and Discussion, Conclusions, Acknowledgments (if applicable) and References.

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Editor's Note

2011 will see our journal entering its fifth year of operation, along with our pride to have served quite a number of researchers around the world in disseminating their work. We are glad our journal can be an outlet for their varied talents and interests and we thank them for choosing our journal to publish their work.

Without all the honoray referees, however, we would not have fared this much either. So we also thank them all for their indispensable service. Their names have been listed on our webpage in acknowledgement of their contribution.

The major problem is that our journal is run on a non-profit basis. This unfortunately means that we cannot grow as much as we want to grow or do as much as we wish to do to serve all our enthusiastic contributors in a satisfactory manner. The time factor is now our big issue stemming from a limited number of personnel that try to serve while maintaining an acceptable standard of a journal. Anyhow, with the possibility of more volunteers we are hoping that things will become better this year.

Duang Buddhasukh
Editor

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Opinion

Who owns science, owns society

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Abstract: The fundamental base underlying scientific empirical principles is currently at high risk of collapse or extinction. Under threat from pseudo-science and technologies which are advancing faster than philosophies can accompany them, and in a grey zone of masqueraded ethical principles, the schools of thought that were once thought to be the omnipotent cornerstones of the scientific institute of knowledge are now doomed to become historical relics. Increasing polarisation of rich versus poor, subsidised versus not, Impact Factor vs non-Impact Factor, paid-view versus open access, and a whole host of other conflicting concepts is further dividing the elite minority of the global scientific community from its silenced majority. Awareness constitutes the first step on the road to constructive change. Shrouded in quasi-ignorance, scientists from Beijing to Belfast are fast being locked in a cycle that may revolutionise the world of science as we know it, blindly, but not painlessly. This small opinion paper is meant to be a nutcracker of sorts intended to initiate a cascade of counter-measures to stem the inevitable tide.

Keywords: empiricism, fallibilism, pragmatism, rationalism

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Note: Terms in ***bold italic*** type indicate new terms coined by the author for the purpose of enlightenment in this article.

AN OVERVIEW

The pillars of society have always redefined themselves over time, either in response to an inductive cue or naturally. A few centuries ago, one would have said that the religious institution and the state (synonymous to religion and the rule of law) would have constituted the two fundamental pillars of society despite its Jeffersonian separation [1]—at least in the Western world, and possibly so within Islamic societies [2]. Law, religion, science and culture have always been the most influential driving factors in moulding societies, and whoever held a reign of power on any one of these—even only one—would most likely succeed in establishing and maintaining a strong (hold on) society. However, unlike natural evolution, social change has been moulded almost exclusively by humans. As currency evolved from trading and bartering and postal mail evolved into e-mail and texting, so too is society unrelentlessly and rapidly evolving. One could almost say that human society has perhaps evolved much more over the past 10-20 years than it has in the past century, if not millennium. Although this claim might be hotly disputed, the philosophical power struggle within and for modern society underlies the changes that science (and thus society) are now undergoing. A recent socio-economic and political assessment of China considers 8 pillars of Chinese society: emancipation of the mind; balancing top-down and bottom-up; framing the forest and letting the trees grow; crossing the river by feeling the stones; artistic and intellectual ferment; joining the world; freedom and fairness; and from Olympic medals to Nobel prizes [3], although a close examination of these (as well as a conversation with the Chinese and a trip to China) would simply indicate that the *id* [4] *Sinica*, ***neo id*** or pluripotent ego is being enhanced with the purpose of capturing the attention of the world and perhaps much, much more. Kuran [5] tries to bring sense to the delay in the economic trend in the Middle East: "Islamic legal institutions, which had benefitted the Middle Eastern economy in the early centuries of Islam, began to act as a drag on development by slowing or blocking the emergence of central features of modern economic life including private capital accumulation, the corporation, large-scale production and impersonal exchange," and "low trust, rampant corruption and weak civil societies—all characteristic of the region's economies today and all legacies of its economic history—will take generations to overcome." A profound assessment of the evolution of society and the role which science plays in that process would be almost impossible. Thus, this small viewpoint takes a broad look at how science has indeed become a power tool in the development and evolution of selected modern societies, independent of culture or creed.

In their day-to-day survival mode, people tend to quickly forget how deeply their lifestyles are determined by, enveloped in, and to a certain extent controlled by science. To understand the depth of this link, one would have to step back and attempt to understand the definition of science. It would then be important to understand how science affects our everyday lives in real, tangible terms. Finally, through my personal interpretations, I wish to show how some sectors of society are strongly determined by science, and how this will to a greater extent affect all scientists and ultimately members of society. This opinion paper will attempt to draw some of those links.

WHAT IS SCIENCE AND CAN IT BE CLEARLY DEFINED?

Most likely many scientists would not be able to provide a concrete or succinct definition for the term 'science', and most likely the definition provided by a scientist would be quite different from that by a non-scientific member of society. Indeed, Gieryn [6] summed up the lack of demarcation between scientific thought and other intellectual activities: "Thus, 'science' is no single thing: its boundaries are drawn and redrawn flexible, historically changing and sometimes in ambiguous ways." The demarcation of science may thus have been equivalent to defining what constitutes quality in science, pure or applied. A broad definition would indicate that science consists of a root system of knowledge bases or truth centres which have emerged from natural laws which themselves have become established from pure and fundamental trial and error. Knowledge in its purest form constitutes science, as would be confirmed by its Latin equivalent, *scientia*. The ability to question an unknown and to attempt to discover the cause of its existence through explanations or predictions would be the defining of scientific principles. Being able to observe or identify, investigate, provide theoretical models and use different methodologies to prove or disprove theories would constitute the scientific methodology at the heart of science, although different schools of thought as briefly and broadly described next would attempt to dissect this basal truth. Much to Aristotle or Pliny's horror, the outcome might not be necessarily logical or rational. Finally, being able to describe what has been challenged and observed constitutes one of the most important elements of the descriptive process underlining empirical science. Effective science writing has led to the historical chronicalisation of a fact, big or small, open to debate and dispute. The person who conducts science even as an art is a scientist or a scientific artist.

More simplistically but very realistically, science is everything and is in everything. Even religion is science and so is art, although not all religious persons or artists are or can be scientists (see brief notes on ***neo***- or ***pseudo-cientistas*** later on).

SCIENCE SCHOOLS OF THOUGHT: SURREAL GROUPING

Empiricism is a philosophical state in epistemological science that claims that knowledge and understanding emerge from experience as perceived by the five senses through which experience leads to evidence, as obtained through methodological experiments, failure and success. In this school of thought, hypotheses and theories are established, and observations lead to concurrence or refutation of what was initially claimed. Markie [7] highlighted the differences between empiricism and rationalism (concept-based thinking) while another school of thought, pragmati(ci)sm, would most likely have tried to find common ground between the two, with Pierce claiming that rational concepts can be meaningful since their reach is beyond the data provided by empirical observation. Most likely what has set in in most laboratories around the world is a blend of Pierce's three forms of reasoning (inductive, deductive and abductive), padded by logical empiricism and phenomenalism. The Rubik's cube would be the Golden Triangle of empiricism, rationalism and pragmatism, being a finite combination of choices where six faces lead to only one end-point solution through a maze of 6^6 permutations. How then can it satisfy the pluralistic basic that the empirical path of scientific thought is based on fallibilism (see next) where one choice would, in theory, tend to infinity?

Pierce's approach presupposes that (1) the objects of knowledge are real things, (2) the characters (properties) of real things do not depend on our perception of them, and (3) everyone

who has sufficient experience of real things will agree on the truth about them. According to Pierce's doctrine of fallibilism, the conclusions of science are always tentative. The rationality of the scientific method does not depend on the certainty of its conclusions, but on its self-corrective character: by continued application of the method, science can detect and correct its own mistakes, and thus this eventually leads to the discovery of truth [8]. Popper's critical rationalism rejects classical empiricism and its birth child, the classical observationalist-inductivist account of science. Popper believed that scientific theories, which are abstract in nature, can only be tested indirectly when their implications are considered. His scientific theory claimed that human knowledge is hypothetical, based on a creative imagination in order to solve problems. Such an imagination would have emerged from socio-cultural settings [9-10].

SCIENCE AND TRUTH: THERE IS NO UNIVERSAL TRUTH

Once we have assumed that science or the study of science is empirical, this would mean that a truth is always open to questioning or falsification (fallibilism). That means that anything and everything that surrounds us might be false, or a questionable truth [11]. This might have evolved from the monism-dualism dichotomy. While the former claims unity in a field of inquiry, stemming most likely from Hinduistic beliefs [12], the latter provides two alternatives while pluralism would provide multiple alternatives. In ontological dualism, the mind (or spirit) and matter are disconnected. This fundamental dichotomy would allow a scientist to seek the truth if the scientist was disconnected from the spirit. Monists, however, feel that reality is constituted by one kind of substance and accordingly there is a potential causal interaction between any one segment of reality and another despite spiritual unity [13, 14]. In a state of dualism, it is possible that we do not have the capacity to extrapolate too much beyond what our senses perceive. So, to say that gravity does not hold objects down would be an inconceivable challenge to Galileo and difficult to comprehend, especially since, when we drop an apple, it does after all 'fall down'. However, if we hold to our empirical definition of science, gravity does not exist, until proved. Since it was proved, it now remains to be disproved and perhaps re-proved once more; herein lies the essence of fallibilism. Popper [9], using the term 'falsifiability', claimed that if a theory cannot, in principle, be 'falsified' (i.e. refuted) by empirical data, then it is not scientific. In Popper's logic, even if an infinite number of positive outcomes at the level of experimental testing can confirm a scientific theory, a single counterexample is logically decisive because it shows that that theory, from which the implication was derived, is false. The term 'falsifiable' does not mean, according to Popper, that something is false; rather, that if it is false, then this can be shown by observation or experiment. Darwin perceived theory as a necessary prerequisite of empirical investigation: "Without the making of theories I am convinced there would be no observation." [15]. Marshall [16] quoted and endorsed Schmoller's belief: "Induction and deduction are both needed for scientific thought as the left foot and the right foot are both needed for walking."

The proof of reality is a miracle of science and if sufficient evidence exists to support a claim, the science of disproof becomes gradually more difficult. Although such a challenge may sound ridiculous to some, it does constitute the basic fundamentals of science and its empirical lifeline. Extrapolated, not everything we see, hear or feel might be what we have been told it actually is. I would suspect that this might be hotly contested by a theologist, who would most likely challenge all

natural science beliefs with metaphysical interpretations. As we observe the scientific institutions that are now increasingly mastering the art of business science, we are forced to look beyond the crafted factors that impose quality and begin to erode away the empirical base of science. That discourse, despite its fundamental links, lies beyond the border of this opinion paper.

SCIENCE AND HUMANS: THE PHILOSOPHICAL BARRIERS

A few hundred years ago, the terms philosophy, science, philosophical science and natural philosophy might have been interchangeably used. Although there has now been a clearer artificial separation into the fields of natural science and philosophy, there is no doubt that all science requires philosophy, but not vice versa. Comte [17], in the evolutionary law of three stages, distinguished positive science from metaphysics and theology and indicated that science used 'reasoning and observation' to establish laws of 'resemblance and succession.' Consequently, not all philosophers need be scientists, although there is a fundamental philosopher in each and every scientist. This article does not wish to delve too much into the details behind artificial categorisation of science, which can be examined or contested on Wikipedia, but will assume it in its broadest form. The empirical nature of science, namely the ability to observe, test and prove or dispute a phenomenon (natural, synthetic or unnatural) overrides the man-made divisions of natural and social sciences. There are different levels of scientific reasoning [18]: 1) the ontological level (assumptions about the nature of reality), 2) the epistemological level (how knowledge is gained and justified), 3) the heuristic level (how problems are framed), and 4) the methodological level (theoretical explanations and their construction). The classification into empirical or formal sciences thus also loses relevance in the broader discussion that follows. A factor explaining why the public at large might not be able to appreciate the extent or importance of genetically modified organisms, stem cell research or cloning, for example, might be ontological dualism [18]. This is because the fundamental ability to assess facts underlying these fields of study might, purely and simply, not exist. In other words, scientific reasoning among the general public at one or all four levels simply does not exist or is hibernating. Despite this, most humans would follow a natural thought ontology: we live in a world of change; change leads to novelty and creativity leads to diversity, indispensable for socio-economic development [19]; novelty drives technological and institutional evolution, but because its nature is unpredictable [9] evolution is temporally unidirectional. Increasing ontological complexity involves causal interactions between several varied entities [20] which overall interact in a non-linear and chaotic way, further limiting predictability and creating the possibility of emergent properties and further novelties, often irreversible [21]. The ability to conceive, for example, that a transgenic plant might contain a mixture of viral, human or fish genes to mass-produce a vaccine, medicine or nutrient might be philosophically impossible to assimilate if histological development had not been accompanied by ontological development alluded to by Pasteur: "...dans les champs de l'observation, le hasard ne favorise que les esprits préparés" (In the field of observation, chance favours only the prepared mind) [22], hence the ravine that lies between science and society and their inter-comprehension.

SCIENCE AND CULTURE: WHERE IS THE SCIENTIFIC GARDEN OF EDEN?

There is a somewhat misinformed perception that somehow science was born in Europe. To provide such a geographic starting point to the birth of science would be like saying the hamburger was invented or created in the USA simply by basing on its level of exposure or consumerism at that moment of time in history. To state that Science was born in the Renaissance or to father its birth on Pliny or Aristotle would be no less than a humanitarian and historical crime. Science owns no mother, is not limited to any crib, nor is it cubed by the laws and restrictions that define quotidian lifestyles. Science as we know it may have had its origins in the first time a rock was struck against the other to create a spark. It may have begun when the sediment of salt water (salt) was added to food to create a new taste, or when a piece of wood was floated and could be used to carry humans or goods on both fresh and salt waters. Science may have begun when someone first remembered their dreams or when they first tried to understand their conflicts with fellow humans. Science has no owner, no origin, no path or destiny. Scientific thoughts and reasoning are held in the minds of humans and their clues and keys are tied up within nature. Pure, basic science involves the purest form of the quest for knowledge. Applied science would then recapitulate what was learnt in pure science in more 'usable' forms in a search for solutions to practical day-to-day problems. Applied science is not a modern concept at all and would have evolved hand-in-hand with pure science, although most likely with a tailing trail. The Neanderthal who struck the rocks and created the spark might have noted that his source of heat, light and possibly cooking instrument could be so easily extinguished by rain.

Therefore, to attempt to doctor the birth of science, its value or its qualitative nature is a vile disregard of its origin and freedom.

SCIENCE, LANGUAGE AND RELIGION: IS ENGLISH THE UNIVERSAL LANGUAGE OF SCIENCE?

Without a doubt one of the most fundamental puzzles for humankind is how all sorts of life came to exist. However, the beauty of science is its ability to unify conflicting theories– evolutionary or religious. Religion, language and culture are inextricably interwoven and perhaps the modern day factors affecting this tapestry are technology and the Internet. Among English-speaking Western scientific culture, there is an incredibly gross misconception that English is the dominating and universal language of science. Plato most definitely would not have agreed 16 centuries ago, and neither would have Boethius, Mendel or Pasteur. Fate would determine that the age of Christian and scientific discoveries would be played out among the European nations, leaving out other major religions such as Buddhism, Hinduism or Islam in their quest for expansion, only to culminate in the eventual global linguistic colonisation by Britain. Since then, English has become an overpowering force of communication and (regretted by some while convenient for others) the leading language for the communication of science. If it were not for the complexities of Chinese characters, Chinese might have been considered the linguistic tool that would accompany the neo-colonialisation now currently in progress, which would have added a 9th pillar to the Naisbitt and Naisbitt theory [3]. However, to elevate achievements using only one language while marginalising others amounts to no less than a neo-usurpation of science of modern-era proportions and a refusal to consider cultural or linguistic background in a serious light, but rather as a dispensable ramification.

WHO OWNS SCIENCE, OWNS SOCIETY

Rees [23] states, "progress in scientific understanding and technology have been synergistic and vital to one another." When we open our fridge and forget to close the door, the detector that beeps away alarmingly is developed, thanks to science. When we eat yoghurt, the fermentation process and lactobacteria that lie therein that give better intestinal functioning are based on science. When we draw money from an ATM and balances, debits and credits are calculated in a split second or when bullish stocks bust on Wall Street in the time it takes to blink an eye-lid, all these and other economic, currency and trading variables are based on empirical scientific principles. Even the bleaching of hair, the miniaturisation of bonsai, or the separation of oil from water are based on scientific facts. Space, the final frontier, has now become the new frontier between science and society. Who owns space or its access and the ability to survive there, owns a treasure chest to countless and boundless unknown wealth—material or intellectual. A lifetime of effort and happiness lost through a greed-filled oil spill brought about by poor scientific principles and saved by oleophiles; economies destroyed by unstable oil prices, unsteady natural reserves and political instability and stabilised by harnessing the power of the sun's rays; the ability to create massive global social networks that can access and inform (or misinform) billions in the time it takes to click a mouse—all of these, without exception, have fundaments in science. Pure science is used to explore and discover. Applied science is used to perfect and expand. Open to use (and abuse), modern (>2010) science has now become not only a street-wise tool, but a kick-the-can toy for the marketing world.

In most of these cases, what we see and what is tangible have emerged from an empirical testing of experimental conditions, leading to a 'techno-socio-eco-econo-logical' development. The knowledge that may have underlain that science may have emerged from a sea of ignorance whose unknowns required correction and re-correction. Such an explanation would feasibly explain a prototype and its subsequent improved versions: the iPod® leading to the iPad®. The hunger for filling that gap of knowledge or encroaching on the void of ignorance is no longer in the scientist, the image of a bench-top worker and laboratory-mongering, coat-clad person. The ***neo-cientista*** or ***pseudo-cientista*** of the 21st century has emerged as an average 'Joe the plumber' who is capable of seeking a market based on the thought-pattern and desire of consumers. Such an emergence of the ***neo-cientista*** would put into total disarray Gould's ideas that anyone who wants to become a scientist must assume the uniformity of laws and processes across time and space [24]. This abridging of empiricism and rationalism, with nuances of instrumentalism—theories as instruments for explaining and predicting phenomena [25]—held by ordinary men and women of society who are street-, web-, tech- and market-wise, merits a new term in the school of thought and in the philosophy of science.

The power of the mass media, whether tweeting on Twitter®, texting on Blackberries®, scrolling down on iPads®, posting on YouTube®, linking socially on Facebook® or scientifically on Linkd®, is posing an existential threat to the very basis of the core concepts of science and scientists, threatening theories that have held for centuries. The power of mass-data collection on megacomputers or an expanded www2 is challenging the limits of puritan scientific thought, relegating scientific philosophy to 'gutter thinking' and elevating pseudo-science, fringe or junk science [26] to Nobel-level hype. Within science publishing itself, the freedom to choose between

rigorously reviewed journals and those that literally publish on submission is feeding the neo-culture of pseudo-science. When simplistic confirmation substitutes refutation, ***neo-*** or ***pseudo-cientistas*** grip comforting beliefs, overgeneralise and seek thought-unifying gratitude through 'YouTubian' resemblances rather than empirical cause-effect thinking.

Without a doubt and without exception, science affects the way we live our day-to-day life. Science stares society in the face from dawn to dusk. Science affects the way in which societies function (or to some extent determines their dysfunctional patterns), and the entities who hold the keys to science will be (already are?) the forces of social and political empowerment of the future. Publishing companies who hold copyright on millions of papers of intellectual achievements and who charge for their access are artful masters of the science chess game at the expense of only one entity: the scientist. Such media giants or possible government agencies may begin a trend in which science begins to slip from the hands of the creators, the true scientists, into the hands of the wrong entities, independent of culture or geographic barrier. Such a power play of science would certainly result in a historical rift between those that believe and those that do not, and between those that have and those that do not—not too unlike the social barrier that once defined medieval societies. What if those entities begin to determine what is good or bad science, what is ethical and unethical science, and what is open or closed science? We must then begin to question who owns science and what power is held within that science, or within society. To slip into a new era of scientific ownership is to enslave science itself and to deprive it from the freedom to question, explore and express the findings that the science forefathers would have liked to see, and see being expressed. To give it incorrect ownership, independent of correct accreditation and acknowledgement, is to doom science to a historical relic and to take one step back in the evolution of humankind.

Who owns science *does* own society, or at least is beginning to do so. That ownership needs to be re-evaluated and carefully monitored, assessed and addressed.

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Note: 'Opinion' is a forum open for authors to express their personal views. Publication does not necessarily mean that *Maejo Int. J. Sci. Technol.* endorses them.

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Invited Article

Weaknesses in publishing: identify, correct and strengthen

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Abstract: Without doubt, one of the greatest barriers in publishing faced by many Asian, African, South American and Middle Eastern scientists is their language-based difficulties. English has most likely become the leading language of scientific publications, even though there are strong but isolated pockets of journals published in local languages. Thus, scientists who are non-native English speakers will find English to be their number one priority, after the scientific content of their manuscripts. This article details my frank interpretation of what I see as being the greatest hurdles that need to be overcome if science writing is meant to be improved other than the scientific content itself, and how to make measured choices that would ensure the best representation of their work. I provide personal, concrete measures and advice on how a young and budding scientist, or even an established scientist mainly from non-English speaking countries, could tackle non-science-related problems. This advice has been formulated from personal experience as a scientist, writer, consultant, editor and director. I hope that it will prove useful to the readers either as a refresher-type 'course' or as a learning experience for those embarking on a new journey into science publishing and writing.

Keywords: publishing, language difficulties, gap in science, love for science

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BROAD CONSIDERATIONS

Back to Basics

Whenever I consider how to write a manuscript for an international scientific journal, I always appear to re-trace my steps back to the first years of undergraduate studies. It seems like the advice I received at that time (detailed below) was the most important tool that would most support the rest of my scientific writing career. Thereafter, through trial and error, and often through painful sacrifices, I began to develop a writing skill which would define my style and sense of interpretation of scientific facts, two factors that strongly influenced the fortunate collection of manuscripts that I have published to date. This paper highlights the main points as I see as being the most important in developing writing skills and strengthening the content of a scientific paper to be submitted to an international journal. There is a wealth of printed and online journals, with and without an Impact Factor®, subscribed or Open Access, that can accommodate almost any level of science as well as taste, creed or culture. My hope is that these guidelines will prove useful and bring budding scientists a step closer to improving their scientific writing skills. To the reader, kindly note that I am not an expert on improving scientific writing skills and the advice that lies herein is based exclusively on my personal experience, primarily as a scientist and as an editor.

Formulate an Idea, Develop a Hypothesis, Grow the Research, Reap the Benefits

An experiment evolves from the birth of an idea. If the idea has no substance, if it is not based on some inspirational seed of thought or does not test a fundamental hypothesis that has not yet been tested, even if small, then the chances of having the study published in a good journal are likely to be slim. So one would have to ask oneself the following questions at the planning stage before beginning the experiment:

a) Has this research ever been published and if yes, was the same material/method used? If not then has the unique value of the manuscript been made clear in the Abstract, Introduction and Results? State the objectives, the hypotheses and what the study initially set out to achieve when the research was started. Always be sure to follow up with future perspectives of the next step in the research somewhere at the end of the Discussion or Conclusions section. This always adds some continuity to what is being done and indicates to the reviewer that there is a broader content to what has been investigated in the study. I always advise my colleagues that not everyone can publish in *Science* or *Nature*, but that each and every one of us has the capacity to identify a unique 'gap' in science where a new discovery could be made. Never shun your own ideas, never be shy about a modest dream to discover something, and never be afraid to ask for help, even if you are the leader of a field of study, in trying to achieve it.
b) If one were to disprove a null hypothesis, then how novel would the results be? Would you consider your own results to be of high international value, high value but geographically localised, or of low practical value or low regional interest?
c) Even if the results are not that interesting or novel, do you have the creative ability to make the story surrounding the data interesting? Even if the methodology used is not that popular, can you still show the effectiveness of simple but powerful techniques? Many top level journals receive

dozens if not hundreds of manuscripts a month and often there is a first phase of selection. During this phase, even if the results are fairly simplistic, provided that they can show some futuristic application or indicate an extension of a concept, the chance of acceptance even with only a single set of data is high. If you feel that something has never been researched before, possibly because the concept would be laughable, ignore those laughs and follow your gut feeling and scientific base. More often than not, you will be the last one laughing.

Based on these three initial assessments, a journal should then be selected that would best correspond to the estimated quality and level of the manuscript's scientific value. Always aim for a slightly higher level journal that you would expect to publish in at first (if time permits) and then downgrade later if necessary. The quality of books is rapidly diminishing and unless the publisher can provide a top-class product on high-quality print medium with a perfect finish, your best bet is to aim for a respectable journal instead.

Experimental Design and Statistical Analyses

A poorly designed experiment or an experimental set-up that has not been well thought of can result in a waste of many months' work in the lab or in the field. I was always told as an undergraduate that for every hour spent in the field (or lab), six hours should be spent at the desk, either analysing data or writing. This is an excellent rule of thumb. Sample sizes or treatment replicates not considered carefully can often result in a journal turning a manuscript away even before it enters the peer review per se. Define the samples clearly including controls, explain the methodology in sufficient detail to allow for the protocols used to be completely reproduced, or if they are based on a previously published protocol, then provide that reference. It is advisable to redefine a protocol using one's own words when it has been published a long time ago or in a difficult-to-access or old journal or source. If detail is not required, the editor will then advise you to remove it later. The choice of statistical analysis will also be fundamental in the interpretation of data and will affect the conclusions that emerge from the work's results. Explain clearly the experimental design used, the statistical treatments and any software that might have been used. Never make the common and grand mistake of matching the analyses to the data set so that the interpretation is swayed in the direction you want it to go. This is unethical.

PERSONAL QUALITIES THAT INFLUENCE THE OUTCOME OF A PAPER

Persistence

It is often said that practice makes perfect. This could not be more true than in scientific writing. You might fail to publish in one journal, but after editing your manuscript more than once you might find that another journal might consider your work to be of value and thus publishable. Do not give up and if you believe that you have a result of interest or practical application, then believe too that there will be a journal that will share your vision eye-to-eye. Improvement of the content can come through discussion with colleagues, peers or supervisors. Try to assimilate as many ideas as possible since this will likely represent the heterogeneity that will exist in the peer review stage.

Publishing is increasingly competitive and as scientists excel more and more at publishing in higher level journals, so too does the possibility of having work published in high level journals diminish.

Desire, Passion or Ambition

There are several reasons why people want to publish: a) they need to publish; b) they would like to publish; c) they have no option but to publish! Naturally, a scientist who has a desire to publish will most probably meet with success because the wish is genuine. A manuscript written because of an artificially-imposed pre-requisite or formal requirement can often result in a poorly written text since there is no real desire to do so. More often than not, scientists who work in a research institute are expected to publish a few papers per year, preferably in high level journals. It is my experience that those who have an innate passion for their subject matter tend to express it very readily in a scientific paper. What the reviewers and editors find from that initial submission is how much passion and care is put in that manuscript. Manuscripts submitted with sloppy style, no attention to detail or in the incorrect journal style tend to create a bad first impression that is likely to linger throughout the entire review process and may affect its outcome. Remember that reviewers and editors are human like you and they all have their regular stresses at home, at university, and so when they receive a poorly written or messily presented manuscript, it is likely to negatively affect their initial (and possibly final) decision. Lower level journals tend to look past these aspects or not consider them too seriously while higher level journals will most likely turn this type of manuscript away a day or two after submission, or at best request the authors to make the necessary edits and re-submit. As editor-in-chief of my journals, the approach is rather unique. Prior to initiating the peer review, there is an intense style and language revision stage. Authors are not or rarely turned away immediately and manuscripts, even those that are riddled with errors, are given a unique opportunity to improve, two or three times if necessary. The philosophy is that human error is inherent and should not be a factor for punishment. However, if the same errors are ignored or not addressed, expect the manuscript to be rejected without entering the review process. On occasion, the manuscript is rejected prior to the review process due to narrow focus, incompatible theme with the journal's objectives or simply because more mechanistic explanations are not provided as required to meet the standards of that journal. In this case, the scientist will have to evaluate the target journal more carefully and reassess the pertinence and effect of the study's results in a broader scientific community. This will of course depend on the inherent knowledge of the topic and the field of study and good experience with a range of journals.

Try to reach a balance between what you have to do and what you want to do. A scientist who is ambitious will more likely pursue a career more strongly than another who does not place so much emphasis on their job, and usually the skills learnt in one manuscript will prove valuable in the next manuscript submission.

Time and Balance

We are all busy, and for most of us (unfortunately), most of the time. This seems to be a new reality as competitiveness and excellence begin to define everything we do in the workplace. We

think that we save time with our new hi-tech trinkets, our blogs and social networking sites, but in fact our time is being cut shorter and shorter. Increasing demand for quality at the work place, more publications and higher level publications, made easier by faster processing speeds, easier software and more user-friendly online submission systems, will all add pressure to an already stressful task of writing the manuscript. A balance needs to be struck between what you have to do and what you want to do, between work and family, between workplace and home. More time can be created by focusing on the important aspects only. A balanced work and personal life will result in a more satisfying writing experience, no doubt.

Confidence and Reliance/Dependence

More often than not, we work in research groups where the emphasis is on group achievements. Even in non-Asian cultures where the individuals, rather than the groups, like to emphasise their own personal achievements, group work is essential. Each person in the lab plays their part, and partners external to the lab play theirs too. This almost invariably implies that each member, corresponding almost always to a co-author of a paper, will have different strengths, functions and responsibilities. When compiling a scientific paper, always be sure to pass the final text to peers and colleagues after all the co-authors have made their suggestions and edits. Never submit a manuscript unless all co-authors have seen, and approved, the final draft. Although too many cooks may tend to spoil the broth, in the case of scientific writing, occasionally more minds will positively influence the quality of the text. Making a mistake of pre-emptive submission may lead to embarrassing situations, which may result in (or actually have resulted from) conflicts of interest and, in worse-case scenarios, ethics violations. Over time, one will learn to appreciate co-authors' comments and will be able to pre-empt errors in submission through experience with previous manuscripts. At some point the learning curve, i.e. ability to write and express in your own way and to submit and respond to reviewers' and editors' queries, will reach a plateau, and at that time you will neither need to read this advice nor rely so heavily upon others to assist you.

WRITING SKILLS THAT INFLUENCE THE OUTCOME OF A PAPER

Basic English Skills

One does not have to be Shakespeare to write a scientific manuscript, but most certainly there should be a good command of the English language. Most international journals publish in English and require that the English be of a high level. If the author is unable to provide such a level of script, then either the assistance of a colleague who is proficient in English or a professional service is often required or requested by the journal. Write formally and write succinctly. Some journals accept that co-authors are included who did not necessarily design or perform the research but who significantly wrote, compiled or improved the manuscript for publication (i.e. at the scientific and linguistic levels). English revision services usually charge in the range of US$ 100-250 for a short communication of 1500-300 words or US$ 200-400 for a research paper of 3000-6000 words. As is evident, most scientists in most non-English speaking countries would not be able to afford such a service, which can amount to a month's salary or more. Therefore, if a scientist is

absolutely unable to write his/her own manuscript and/or feels that inviting a high level scientist as a co-author and thus team member would significantly improve the quality of his/her text, then do so. I am of opinion that there is nothing unethical about this decision and that it may give a scientist—even an established one who has reached a plateau in writing ability—greater confidence through learning as to how to better improve writing skills until he/she is confident enough to complete this task on his/her own. Always make sure, however, that this agreement does not result in conflicts of interest with co-authors, with the research institute where the research is conducted or with funding bodies. This is a complex and highly sensitive topic and can be the subject of another separate writing. Such co-authorship collaboration is currently a grey zone in science publishing, and to clarify this we need to approach science publishing in a step-by-step manner.

Whenever I establish an international co-operation of this type, I always set out the most rigorous ethical policies possible and request all of my co-operating partners to agree to every single point, without exception. Note that these are only guidelines and should be set to meet the individual requirements of each research group or study.

Declaration of Ethics of Co-operation:

a) This co-operation exists with an understanding that this work is original, has not been published before, has been executed in the most scientifically rigorous manner possible, and has not been considered for more than one journal.
b) This co-operation exists with an understanding that there are no conflicts of interest between any of the co-authors or any of the authors and research institutes and/or funding bodies.
c) Any possible conflicts of interest that have not been fully and openly declared are and will be the FULL responsibility of the host research author(s) and institute.
d) Submission is also the FULL responsibility of the host research author(s) unless specifically requested due to difficulties with language or complexity of online submission systems. In that case, I will serve only as the vehicle for submission but the original authors will remain the official authors for correspondence in the manuscript itself.
e) The functions performed by me (upon request) include advice on data analysis, advice on experimental design and analysis, critical assessment and evaluation of scientific content, and language improvement, each to a different extent but all-inclusive nonetheless.
f) It is understood that such a co-operation is NOT standard BUT constitutes a unique, but not unethical, means of co-operation, as established by all parties concerned. Independent of this co-operation, the ethical guidelines set out by relevant journals, research institutes, funding bodies and publishers will be fully respected.
g) Submission of this manuscript to any and all future journals implies that all conditions a) to f) have been read by all parties and are fully understood.
h) To ensure that all conditions are met, each revision and submission step will be communicated to all authors by e-mail. This is done to avoid any misunderstandings or conflicts and to promote full transparency and open communication and discussion at each step of the editing process.

i) The choice of journal, publisher and publishing medium (online or print, journal or book), format (open access or paid subscription), and level of quality (with or without Impact Factor®) will be decided upon by consensus.

Failure to meet even as much as one of these requirements will result in the co-operation not taking place due to conflicts of interest, personal or professional.

Never Assume Eloquence and Talent

One of the basic flaws in many manuscripts that are submitted by researchers for the first time, and indeed by experienced researchers too, is the assumption that what they have written is correct and logical. No matter how many times a scientist reads his/her own manuscript and no matter how convinced he/she is that the content is correct and good, there will always be errors somewhere! We always observe things in a subjective way through our own perspective, and often it is useful to sit together with the co-authors to thrash out better structured sentences, more concise phrases or clearer concepts, better understanding and interpretation of the data. If English is not a scientist's first language then it is always advantageous to improve writing skills through training exercises, although there might not necessarily be a correlation between English and scientific English skills. Even if a scientist is a leader in a field of study, there will always be different interpretations held by each person (editor or reviewer) who reads the manuscript, and this may be the case even after it has been published, so it is important to eliminate ambiguities early to avoid confusion (in the mind of the reader) later.

Think Like an Editor and Reviewer

Whenever submitting a manuscript to an international journal, always expect there to be about three levels of revision: linguistic, stylistic and scientific. Each journal or publisher will have different approaches and levels of importance assigned to each of these. In the journals for which I am editor-in-chief, there is an initial screening of the manuscript. If the authors have bluntly ignored the instructions for authors and the guidelines for submission of a manuscript, then the manuscript is immediately returned for correct formatting. If the manuscript is correctly formatted but the text is too unclear or poorly written (language-wise), it is returned for linguistic improvement. To save time (author's and journal's), close attention should always be paid to the style and requirements of the journal. Write a good covering letter, but do not be verbose. State only what needs to be stated. Do not just copy-paste the abstract into the letter; that will surely irritate the editor/reviewer. Remember that in most cases editors and reviewers are humans too, and that they have their careers, their responsibilities and their personal lives. In general, they are busy and they do not have time or patience for errors or messy submissions. Therefore, a manuscript that is incorrectly formatted, poorly written, with messy presentation or with weak language skills will most likely receive a negative response almost immediately. Half of the success of an accepted publication is a manuscript that takes these very basic requirements into consideration, which would leave the editor and/or reviewer the space to simply focus on the scientific content. So before submitting, have a long, good look at your manuscript and ask yourself: Am I happy with the quality of this submission? And will

the editor and reviewer who receive it be initially satisfied with the quality and willing to read and assess further? The reader might easily interpret my call to think like an editor and reviewer as being a request to conform to a standard manner and style of writing. Not at all. I strongly encourage authors to adopt their own style of writing wherever possible, alone or with the assistance of a professional service or appropriate co-operating partner.

Graphics and Tables

Remember always that figures and tables are meant to support the text and not repeat any data within it. So always provide these in a way that will strengthen the content of the text. Basic and simple things like defining all abbreviations in the table footer or figure legend, showing error bars, *P* values or the test of significance employed are actually essential pieces of information that makes the table or figure independent of the text. Photos that have poor resolution, graphs that have different font sizes and styles, and tables that look messy and with poorly organised data are all ice-bergs waiting to sink your manuscript entitled "The Titanic."

SKILLS IN DEVELOPING THE INTRODUCTION AND DISCUSSION

Synthesise and Paraphrase

Synthesis of information is vital for crunching a mass of information into a limited space. Most journals are getting stricter and stricter about word count, size of sections and manuscripts themselves. The capacity to synthesise thoughts and data is thus one vital aspect to the success of the outcome of the review process.

Occasionally we find text that is so well written and that perfectly describes what we want to say. If those words are used directly, then they should appear in quotation marks, and a general rule of thumb is never to quote more than 100 words to avoid copyright infringement. If there is a fear of such an infringement or the journal cannot provide clear and suitable guidelines, then avoid direct quotes. In this case, try to reduce what has been said in a few lines, i.e. paraphrase the concept so as to capture the essence of the idea, remembering always to reference the source. If due recognition to the words or ideas from another source is not given, this constitutes plagiarism, which is an ethically grave publishing error. This topic can also be dealt with separately in more detail in another paper.

Speed-read

When writing an invited review, for example, one often has to sift through dozens if not hundreds of articles to find information that would strengthen the review within the shortest possible time. Since time is always the limiting factor, and unless there is an assistant, the best way to cover so much literature is through speed reading. When trying to find suitable references to build the Introduction or references to support the findings in the Discussion, speed-reading is a very helpful technique. There are no hard and fast rules, and such an ability comes with practice and years of experience.

Link to Your study: Relevant and Up-to-date

Whether the Discussion is separate or joined to the Results section, always be sure to reference any studies from the literature that are directly (or closely) linked to your study or to its results. Do not add masses of references just because you think this will impress the reviewer. The reviewer will be more impressed if you reference less but show the links more strongly using pertinent studies. Always remember to provide an updated reference list, so check major data-bases just before submission. Check the limits for section length and reference numbers but a safe guideline would be a roughly equal size for each section.

THINGS NEVER TO DO IN A SCIENTIFIC PAPER

Although the following comments may appear obvious, there is surprisingly a high number of manuscripts, even to top level journals, that breach one or more of the following:

Never falsify data. Never plagiarise. Never self-plagiarise. Never simultaneously submit manuscripts that have already been submitted to another journal. Never submit data that pose a conflict of interest with colleagues, financial institutions or your research institute. In co-operation settings, it is always best to follow the basic rules as described above in "Declaration of ethics of co-operation."

Always conduct research and submit with the highest possible level of ethics. Remember that there are also now easier ways of digitally tracking manuscripts and information within them and to make an ethical error could jeopardise your career and taint your name and the name of your institute.

SUMMARY AND CONCLUSIONS

Writing a scientific paper for an international journal is for some a pleasant experience while for others it is a stressful one. Most certainly it will contain both aspects but hopefully within the text above you may find at least one point that might lead you closer to having your important research results published more easily and more effectively. Remember that a manuscript which is well written (style-wise) and contains clear and grammatically correct English, which follows the style of the journal and proves the hypotheses initially set out in the Introduction will have already fulfilled half of the requirements for publication in any journal. The remainder depends on the quality of the research that was conducted, on the scientific merit and uniqueness and on the strictness of the review process.

Note always that quality (as in the 'quality' of a manuscript) is often made into an objective (factual) parameter by each journal based on specific guiding principles and selection criteria, but it is often implemented by editors and reviewers who provide, to some extent, a subjective (personal) view, even if underlying.

I wish you well in your professional writing endeavours.

ACKNOWLEDGEMENTS

I wish to thank Pham Thanh Van for critical opinions and for support in turbulent times. This text is dedicated to all those scientists who inspire me with their passion to make it happen. This message is for you: "Rise and rise again until lambs become lions" (Robin Hood, 2010).

ANNEXE

Helpful statistics sites:

SurfStat Australia: http://www.anu.edu.au/nceph/surfstat/surfstat-home/surfstat.html

STEPS: http://www.stats.gla.ac.uk/steps/index.html

HyperStat: http://davidmlane.com/hyperstat/

The Mesa Cohort: http://glass.ed.asu.edu/stats/

Virtual Anesthesia Book: http://www.virtual-anaesthesia-textbook.com

Useful sites for practicing English and improving grammar:

http://www.shared-visions.com/explore/english/ http://owl.english.purdue.edu/handouts/grammar/

Other similar resources but with more formal structure:

http://www.cumc.columbia.edu/dept/gsas/ac_programs/writing.htm

http://www.ajronline.org/cgi/content/full/188/5/1179

http://codecourse.sourceforge.net/materials/Writing-Research-Papers-Collected-by-Nakov.doc

http://www.ruf.rice.edu/~bioslabs/tools/report/reportform.html http://www.word-medex.com.au/formatting/index.htm

http://www.columbia.edu/cu/biology/ug/research/paper.html

http://www.sciencebuddies.org/mentoring/project_research_paper.shtml

Plagiarism:

http://www.ehow.com/about_6368008_description-constitutes-plagiarism.html

http://plagiarism.org/

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Full Paper

Evaluation of morphological diversity in south Indian tea clones using statistical methods

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Abstract: Morphological diversity of three *Camellia* (Theaceae) taxa conserved in an ex situ gene bank was studied and the importance of different descriptors in categorising accessions into distinct groups was also determined. Twelve accessions were characterised using 15 morphological descriptors of IPGRI guidelines. The results of principal component analysis (PCA) on morphological characters showed that the first two principal components accounted for 44.77 % of the total variance. In the evaluated quantitative characters, all three taxa had a coefficient of variation (CV) greater than 24.85%, and within the taxon the CV was greater than 9.59%. The qualitative characters showed a wide range of variations and yielded significant differences ($p<0.05$). Phenotypic data had high contributing component loadings from characters such as leaf area, weight of harvested shoots, stem colour, leaf pubescence and young shoot colour. Cluster analysis delineated the accessions into three groups. The implications of our results hold promise for assessing genetic diversity in germplasm collections, which is a prerequisite for their utilisation, effective management and crop improvement.

Keywords: *Camellia* spp., morphological diversity, numerical descriptors, tea germplasm

INTRODUCTION

Tea [*Camellia sinensis* (L.) O. Kuntze] has been known for more than 2000 years in China and is naturally distributed throughout tropical Asia [1]. Tea production in India started in Assam in 1823 by Robert Bruce. South Indian tea has diverse genetic resources, since all existing plantation stocks are the progeny of the plants or seed stocks brought from Assam, China and other sources. Initially, seed stocks were imported from China through Kolkata botanical garden and planted in Nilgiris in 1832 for experimental purposes [2]. Subsequently, a few more plants from Assam and China were also planted in different areas in southern India. Later, the tea scientific department of united planters association of southern India (UPASI) produced accessions from natural populations and also through breeding methods [3]. The genetic resource of tea in UPASI is undoubtedly one of the most important sources of tea germplasm resources in India. A large number of controlled hybridisation were attempted and some of the progeny were also recommended for planting [4]. The existing diversity will have to be preserved and characterised for future crop improvement programmes that constitute the fundamental support structure for the tea industry.

Leaf morphology has an important role in identifying taxa in which variation in floral structures is uninformative or in which flower specimens are infrequent owing to a limited flowering season, for example [5]. The use of morphological characters is cost-effective when compared to that of biochemical and molecular markers for preliminary characterisation of many individuals to identify morphologically similar groups and for simple varietal identification of phenotypically distinguishable cultivars [6]. In tea, morphological characters have been used to study genetic diversity [7-8], variation [9-11], phylogeny and classification [12-15]. Leaf features have been largely unexploited in taxonomic studies, resulting from a belief that they respond in a plastic manner to environmental factors. However, in ex situ gene banks, the plant materials are grown under similar environmental conditions and farming practices, making it possible to compare taxa.

Statistical methods have been reported [16-18] and there are two main types of techniques to represent taxonomic structure: cluster analysis and principal component analysis (PCA). It should be useful for both the breeding programme and the germplasm conservation of tea plants to understand the diversity and differentiation of morphology among those taxa. In the present study, cultivated tea clones of *Camellia sinensis*, *C. assamica* and *C. assamica* subsp. *lasiocayx* in south Indian germplasm are morphologically described and assessed for their diversity by applying statistical methods. The aim is to detect intra-specific boundaries and to identify reliable distinguishing characters. This study will provide a basis for further investigations of systematic classification using the data from morphological characters.

MATERIALS AND METHODS

Plant Materials

Twelve tea cultivars belonging to *Camellia sinensis* (China type), *C. assamica* (Assam type) and *C. assamica* subsp. *lasiocalyx* (Cambod type) were collected from the UPASI-TRI (United Planters Association of Southern India - Tea Research Institute) germplasm collection centre at Valparai, Tamil Nadu (Table 1). Five randomly selected plants from one plot of each accession were

used to record observations on morphological characters. Young shoots from each cultivar with two leaves and a bud fully exposed to sunlight were collected and morphologically described. Fifteen important characteristics of stem, 4th leaf and young shoots were analysed qualitatively and quantitatively following the guidelines of IPGRI (International Plant Genetic Resources Institute) [19]. For characterisation, IPGRI descriptors were adopted. A total of 17 characters were scored. For each specimen, five mature, healthy looking leaves were scored and averaged. Upon further examination, it was found that two characters were constant (not informative) and were eliminated from the analyses. Finally, 15 characters were selected (Table 2). In our study, due to the homogeneity of local climatic conditions and the similarity of farming techniques applied, the effect of those factors on the morphological characteristics was not considered or interpreted. For quantitative characters, coefficients of variation (CV = standard deviation / mean) among the clones at inter- and intra-specific levels were calculated. A Kruskal-Wallis test [20] was used to determine the differences of qualitative characters among the three taxa.

Table 1. List of evaluated *Camellia* stock and their clones

Camellia species and their clones (type)	Accession number	Source of material
Camellia assamica (Assam)		
'UPASI-2'	B/ 4/142 (Jayaram)	Brooklands Estate, the Nilgiris
'UPASI-3'	B /5/63 (Sundaram)	Brooklands Estate, the Nilgiris
'Assam Seedling'	--	Assam
Camellia sinensis (China)		
'UPASI-9'	B/6/61 (Athrey)	Brooklands Estate, the Nilgiris
'UPASI-10'	B /6/62 (Pandian)	Brooklands Estate, the Nilgiris
'TRF-2'	NLT/17/10	The Nullatanni Estate, Munnar
'SA-6'	--	High Wayves, Tea Estates India
Camellia assamica subsp. *lasiocalyx* (Cambod)		
'UPASI-17'	B /6/203 (Swarna)	Brooklands Estate, the Nilgiris
'TRF-1'	Selection A	Arrapetta, Wynaad
'CR-6017'	--	Craigmore, the Nilgiris
'BSS-1'	Biclonal seed stock	UPASI-10 x TRI-2025
'TRI-2025'	--	TRI, Sri Lanka

Principal Component Analysis

To explore the pattern of variations in measured characters and to find those decisive characters for distinguishing taxa, PCA was carried out using mean values of morphological observations. PCA can be used for transforming attributes of a dataset into a new set of uncorrelated attributes (principal components) while still retaining as much of the variability of the dataset as possible. It can also handle variables of different types (nominal, ordinal and numerical)

simultaneously and deal with relationships between variables. In addition, Cronbach's alpha [21] was calculated for each of the components extracted.

Cluster Analysis

Clones of the three *Camellia* taxa were grouped by cluster analysis using the unweighted pair group method analysis (UPGMA) based on the similarity matrix of Euclidean distances of the morphological data. To trace the relationship among the tea clones, the data were standardised before clustering and a dendrogram was constructed. The statistical analyses were performed using the STATISTICA software version 4.5.

RESULTS AND DISCUSSION

Morphological Diversity and PCA

The variation in morphological characters of *C. sinensis*, *C. assamica* and *C. assamica* subsp. *lasiocalyx* is summarised in Table 2 by applying statistical methods. Multivariate statistical techniques such as PCA and cluster analysis are commonly used methods for characterisation and genetic diversity analysis of germplasm and can increase the accuracy of interpretation of information generated in characterisation studies [14]. Characters were chosen with respect to variations among taxa mentioned in the literature and also based on careful observation of specimens. The PCA results on morphological characters showed that the first two components accounted for 44.77% of the total variance in the dataset (Table 2). The principal component of single trait accounted for 29.84% of the total variance and was highly interpretable (Cronbach's alpha = 0.80). If the alpha value of a specific component is high, it is interpreted as indicating that the component has a strong one-dimensional structure, or the dimension can reliably account for the total variance. Generally, an alpha value of 0.70 or greater is considered to be reliable [22]. Phenotypic data that had high contributing component loadings were from such characters as leaf area (0.88), weight of harvested shoot (0.83), stem colour and leaf pubescence (over 0.7), and young shoot colour with negative loading (-0.71).

A morphometrical analysis of leaf morphology is a useful and rapid method for identification of species [15]. Morphometric studies on *Taxus* (Taxaceae), a taxonomically complex genus with many sterile specimens like *Camellia*, showed that leaf characters are a powerful tool in separating and identifying species in this morphologically labile plant group [23-24]. Pi et al.[15] investigated 54 species of *Camellia*. In their study, PCA results accounted for 63.2 and 20.6% of the total variance for component 1 and component 2 respectively. The sum of the two components accounted for most of the total variance, whereas in our study, 44.77% of the total variance was observed in the data set with a sample size of three *Camellia* taxa comprising 12 clones. In their report, the average values of lamina vertical length, horizontal width, width-length ratio, leaf area and leaf veins were transformed before they were used for PCA. The results of this study showed that the qualitative and quantitative characters with high component loadings are in conformity. Su et al. [11] compared morphological characters of *Camellia sinensis* (*formosensis*) and two closely related

Table 2. Variation in morphological characteristics of investigated tea samples. Loadings of the morphological characters on first two components are from PCA. Eigenvalues, percentages of variance explained and cumulated, and Cronbach's alpha are given for each component.

Character	Range of variation (Standard deviation)	Data type	Component	
			1	2
Weight of harvested shoot (g)	1.23 (0.343) - 3.70 (0.500)	Quantitative	0.83	-0.01
Leaf area (cm^2)	18.5 (1.000) - 46.5 (6.782)	Quantitative	0.88	0.04
Internodal length (cm)	2.20 (0.158) - 5.50 (0.454)	Quantitative	0.65	0.02
Petiole length (cm)	0.25 (0.500) - 0.50 (0.084)	Quantitative	0.56	0.26
Leaf serration density (number/cm)	8.00 (0.836) - 20.0 (1.140)	Quantitative	0.59	-0.70
Stem colour	Green – dark green	Multistate	0.71	-0.29
Immature leaf colour	Yellow green – green	Multistate	-0.42	-0.54
Mature leaf colour	Yellow green – green	Multistate	0.13	-0.14
Leaf blade shape	Ovate-elliptic	Multistate	0.32	0.61
Leaf apex shape	Acute-obtuse	Multistate	-0.05	-0.38
Leaf blade pubescence	Sparse – intermediate	Binary	0.70	0.16
Leaf blade base shape	Obtuse – acute	Binary	0.20	-0.61
Leaf waxiness	Absent – present	Binary	-0.25	-0.59
Petiole colour	Green – yellow green	Multistate	0.20	-0.12
Young shoot colour	Green - yellow green	Multistate	-0.71	0.14
		Eigenvalue	4.48	2.24
		Variance explained (%)	29.84	14.93
		Variance cumulative (%)	29.84	44.77
		Cronbach's alpha	0.80	0.55

taxa of Taiwan native wild tea plants using numerical methods. In their studies, characters with high loadings were bud pubescence, young branchlet pubescence, abaxial midrib pubescence and petiole pubescence (over 0.7). Thus, significant values of the Cronbach's apha and character component loadings in our investigation are on a par with their findings. Similarly, Hu [25] used 15 leaf characters measured on a tea germplasm collection of Taiwan to evaluate inter-taxa variations among *C. sinensis* var. *sinensis*, *C. sinensis* var. *assamica* and *C. sinensis* var. *formosensisi*.

The evaluated morphological qualitative characters showed a wide range of variations and yielded significant differences ($p<0.05$) among the three taxa (Table 2). Among the qualitative characters, stem colour ($n = 12$, $p = 0.05$) and young shoot colour ($n = 12$, $p = 0.01$) exhibited significant differences. Similarly, significant qualitative characters showed high factor loadings in the first component of PCA (Table 2). Quantitative and qualitative characters showed that clear morphological differences exist within and among taxa in our study (Tables 2-3). Chen et al. [12] reported significant differences ($P<0.05$) in the seven qualitative characters of *C. sinensis* (cultivated tea) and its wild relatives in Yunnan province, China.

Table 3. Variations of quantitative morphological characters of the three *Camellia* taxa

Camellia **Species and their clones**	**Weight of harvested shoot (g)**	**Internodal length (cm)**	**Leaf area (cm^2)**	**Petiole length (cm)**	**Margin serration per cm**
Camellia assamica					
UPASI 2	2.72 ± 0.370	3.1 ± 0.587	46.5 ± 6.782	0.5 ± 0.083	9 ± 1.581
UPASI 3	3.56 ± 0.531	5.5 ± 0.545	36 ± 3.847	0.5 ± 0.122	20 ± 1.141
ASS SEED	3.7 ± 0.370	3.75 ± 0.336	45 ± 1.581	0.4 ± 0.070	18 ± 1.581
	3.3 ± 0.503	4.10 ± 1.217	42.4 ± 5.907	0.46± 0.052	15.5 ± 5.714
C V (%)	15.25	29.65	13.94	11.5	36.78
Camellia sinensis					
UPASI 9	2.15 ± 0.223	4.5 ± 0.791	22.5 ± 1.322	0.4 ± 0.083	13 ± 1.581
UPASI 10	1.53 ± 0.482	3.35 ± 0.414	32.3 ± 1.923	0.3 ± 0.1	16 ± 0.707
TRF 2	1.81 ± 0.114	2.2 ± 0.158	32.2 ± 1.351	0.5 ± 0.071	8 ± 0.836
SA 6	2.29 ± 0.614	3.5 ± 0.371	26.3 ± 1.188	0.3 ± 0.072	15 ± 0.707
	1.95 ± 0.334	3.4 ± 0.942	28.3 ± 4.761	0.4 ± 0.094	13 ± 3.652
C V (%)	17.21	27.72	16.82	25.54	28.2
Camellia assamica subsp. *lasiocalyx*					
CR 6017	1.25 ± 0.343	3 ± 0.547	24.5 ± 0.901	0.2 ± 0.05	12 ± 0.707
UPASI 17	1.97 ± 0.632	3.25 ± 0.207	30.6 ± 1.673	0.5 ± 0.071	9 ± 0.836
TRF 1	1.23 ± 0.343	3.55 ± 0.671	18.5 ± 1	0.4 ± 0.071	9 ± 0.707
TRI 2025	1.57 ± 0.279	2.95 ± 0.23	22.1 ± 0.831	0.3 ± 0.044	16 ± 0.707
BSS 1	1.65 ± 0.594	2.75 ± 0.261	28.3 ±1.404	0.3 ± 0.07	15 ± 0.836
	1.54 ± 0.303	3.13 ± 0.299	24.8 ± 4.821	0.35 ± 0.097	12 ± 3.364
C V (%)	19.72	9.59	19.44	27.64	27.57
Total	2.12 ± 0.825	3.45 ± 0.857	30.4 ± 8.736	0.38 ± 0.096	13.3 ± 3.961
C V (%)	38.96	24.85	28.74	24.68	29.71

Results in Table 3 show that differences, i.e. CV, in morphological characters according to IPGRI guidelines exist among *C. sinensis, C. assamica* and *C. assamica* subsp. *lasiocalyx*. In the evaluated quantitative characters, all three taxa have a CV greater than 24.85%, and within a taxon the CV is greater than 9.59%. Weight of harvested shoot shows the highest percentage of CV (38.96) and the lowest is in internodal length (24.85%). The internodal length character exhibits the lowest CV (9.59%) in *C. assamica* subsp. *lasiocalyx* and the highest is in margin serration of *C. sinensis* (36.78%). Among the three *Camellia* taxa, 28.74% diversity is detected in leaf area. Chen et al. [12] presented the variation in the morphological characters of *C. sinensis* var. *sinensis*, *C. taliensis* (W. W. Sm.) Melc., and *C. sinensis* var. *assamica* in Yunnan province, China. In their study, quantitative characters exhibited a CV of more than 20% in leaf area, weight of harvested shoot, period of flowering, pericarp thickness and seed weight across all three taxa. Incidentally, variations in such characters as leaf area and weight of harvested shoot seem to support our findings. Similarly, Yu and Xu [26] evaluated diversity in tea germplam resources of China using morphological characters. In our study to estimate the diversity in the current UPASI-TRI

germplasm collections (Tables 2-3), the diversity (CV) among the three *Camellia* taxa based on morphological characters is between 24.85-38.96%. Within taxa it is 11.5-36.78% for *C. assamica*, 17.21-27.72% for *C. sinensis*, and 9.59-27.64% for *C. assamica* subsp. *lasiocalyx* (Table 3). This is much lower than results from other studies based on amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) markers.

Vegetatively propagated clones began to replace seed propagation in the 1960s and probably reduced the genetic diversity within tea cultivation [27]. Wachira et al. [28] reported that 72% of the variation resided among individuals within populations of *C. sinensis* and its wild *Camellia* relatives based on RAPD and AFLP markers. Kaundun and Park [29] stated that 16% of the total diversity of RAPD-PCR markers was observed among populations of Korean tea. Balasaravanan et al. [2] assessed the genetic diversity among tea cultivars from southern India using AFLP markers and found a narrow genetic diversity (less than 37.76%), which supports our findings. Significant variations occurred for the quantitative characters among the investigated taxa. Each clone was relatively distinct from one another in their phenotypic characters by multivariate analysis. Hu [25] used 15 leaf characters measured on 132 tea germplasms to evaluate inter-taxa variation among *C. sinensis* var. *sinensis*, *C. sinensis* var. *assamica* and *C. sinensis* f. *formosensis*. Further, there were attempts to study *C. sinensis* and its closely associated species based on numerical methods [30-31]. However, these studies aimed at exploring the variation among populations of *C. sinensis* and its closely related species rather than solving the fundamental taxonomic problems.

In our study we have summarised the morphological variation among the three *Camellia* taxa by applying statistical methods. We have found the intra-specific boundaries and reliable distinguishing morphological characters. A sound knowledge of taxonomy is a prerequisite for the success of any germplasm conservation programme of tea plants. The results summarised here using morphological descriptors of UPASI clones can form a basis for further identification and selection of elite clones from the existing tea germplasm for tea improvement programmes.

Cluster Analysis

A dissimilarity matrix based on Euclidian distances for the three *Camellia* taxa is presented in Table 4. The clones of the *Camellia* taxa exhibited a large variation between and within the taxa. The estimated dissimilarity of 4.58% was highest between the clones UPASI 2 (Assam tea) and CR 6017 (Cambod type) while the least dissimilarity (2.00 %) was between two China clones, namely SA 6 and UPASI 10. A phenogram based on the Euclidian distances from the morphological data divides the section *Camellia* into three clusters, viz. cluster C_1 and sub-clusters SC_1 and SC_2, which can be recognised as *Camellia assamica* (Assam type), *Camellia sinensis* (China tea) and *Camellia assamica* subsp. *lasiocalyx* (Cambod type) respectively (Figure 1). This type of clustering pattern is generally consistent with existing knowledge on the morphology and systematics of *Camellia* species [2, 32-34]. The clustering of tea clones is also in congruence with a recent report of Roy and Chakraborty [35] based on ISSR markers.

Table 4. Dissimilarity matrix of 12 tea clones based on morphological characters

UPASI 2	0											
UPASI 3	2.83	0										
Assam Seedling	3.16	3.16	0									
UPASI 9	2.83	4.00	3.16	0								
UPASI 10	3.74	4.24	4.00	3.16	0							
TRF 2	4.24	3.74	4.00	3.74	2.83	0						
SA 6	3.74	4.24	4.00	3.16	2.00	2.83	0					
CR 6017	4.58	4.12	4.36	3.61	3.32	2.65	3.87	0				
UPASI 17	4.00	4.00	3.74	3.46	4.00	2.83	3.46	3.00	0			
TRF 1	3.46	3.46	3.74	3.16	3.46	2.83	2.83	3.87	3.16	0		
TRI 2025	3.61	4.12	3.87	3.32	2.24	3.32	3.00	3.74	4.12	3.00	0	
BSS 1	3.74	4.24	4.00	2.83	3.16	3.74	3.16	3.00	3.46	3.16	3.32	0

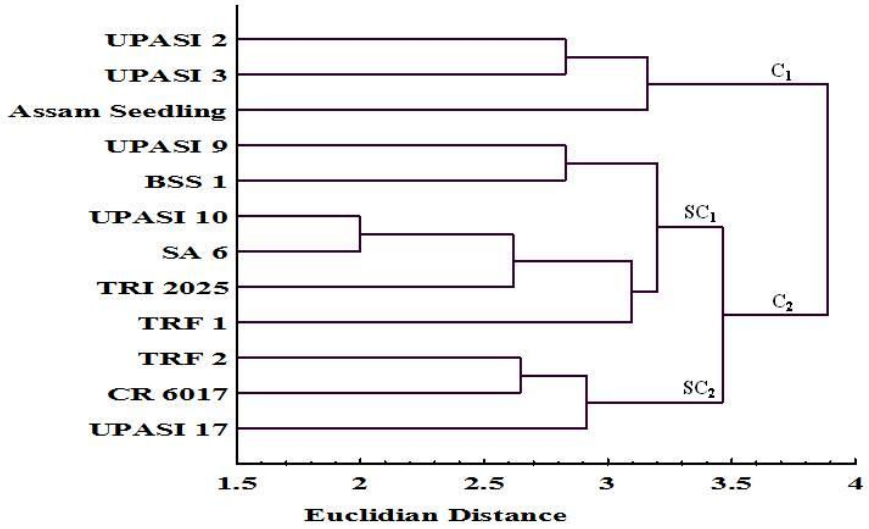

Figure 1. Dendrogram of the 12 tea clones based on Euclidean distances of morphological characters

Cluster 1 (C_1) consists of clones of only one species, *C. assamica* (UPASI-2, UPASI-3 and Assam seedlings), and cluster 2 (C_2) contains clones of two remaining taxa. Thus, C_2 has two subclusters, SC_1 and SC_2, with clones of *C. sinensis* and *C. assamica* subsp. *lasiocalyx* respectively. Subcluster SC_1 comprises China type (*C. sinensis*) clones, namely UPASI-9, UPASI-10 and SA 6. The results obtained in our study confirm the report of Saravanan et al. [36], in which the total leaf catechins and their fractions were used for genetic diversity studies. The clone TRF 2 of Cambod type (*C. assamica* subsp. *lasiocalyx*) in subcluster 1 is an exception. Morphological and molecular studies revealed that TRF 2 should be grouped under China varieties [37]. In the cluster SC_1, along with China teas, hybrid tea BSS1 and TRI 2025 clones of Cambod tea are also grouped.

Interestingly, UPASI 10 and TRI 2025 are female and male parents of the hybrid BSS 1 respectively, developed by UPASI-TRI. BSS 1 is morphologically more similar to Cambod tea clustered in the group of China teas. These results confirm the morphological explanations and

genealogical data of the hybrid and its parents as reported by Satyanarayana and Sharma [4] and Mohanan and Sharma [33]. In our earlier report, the BSS 1 hybrid tea clustered in the China tea group [34]. Vo et al. [13] reported a similar kind of clustering; a hybrid between Indian and Chinese clones (LDP1) clustered with its morphologically similar mother (PH1), which is *C. sinensis* var. *assamica* (Indian tea).

Subcluster SC_2 has Cambod tea clones CR 6017, UPASI 17 and TRF 2 of *C. sinensis* (China type). Clustering of certain clones in our study coincides with results of Paul et al. [38], in which the clone UPASI 17 is grouped under the Cambod type based on genetic diversity studies. All tea clones form three groups in our study based on a statistical methods. Clone TRF 1, grouped in the China cluster along with TRI 2025, is an exception. TRF 1 is similar to TRI 2025 based on morphology [39]. In our earlier study using RAPD marker, the clustering of TRF 1 was found to be in the Cambod group along with TRI 2025 [34]. Vo et al. [13] studied the morphological diversity of Vietnamese tea at Lamdong and produced similar results in the grouping of some clones.

CONCLUSIONS

The present study has shown that phenotypic characters such as leaf area, weight of harvested shoot, stem colour, leaf pubescence and young shoot colour can be used to distinguish between the three *Camellia* species and their accessions into well-defined phenotypic groups similar to the genetic diversity determined by RAPD markers. Therefore, information on morphological diversity should also be useful for future breeding programmes as well as for proper conservation of genetic diversity in the adapted germplasm.

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Full Paper

Effects of alternative protein sources on rumen microbes and productivity of dairy cows

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Abstract: This experiment was conducted to investigate the effect of various protein sources on digestibility, rumen fermentation, milk yield and milk composition in dairy cows. Four Holstein Friesian native crossbred cows in early lactating were randomly assigned according to a 4x4 Latin square design. The dietary treatments containing different protein sources in concentrate diets were soybean meal (SBM), cassava hay (CH), *Leucaena leucocephala* (LL) and yeast-fermented cassava chips (YEFECAP), with ad libitum intake of urea-treated rice straw. Digestibility of DM, OM, NDF and ADF was not different among treatments ($P>0.05$) while CP digestibility was highest ($P<0.05$) in CH and YEFECAP supplemented groups. Ruminal NH_3-N and BUN concentrations varied among protein sources and were highest in SBM and LL fed groups ($P<0.05$). Ruminal total volatile fatty acid (VFA) and propionic acid were found highest in cows receiving CH and YEFECAP ($P<0.05$). Ruminal fungi, proteolytic and cellulolytic bacteria were highest when YEFECAP was supplemented. Milk fat and milk protein were significantly increased ($P<0.05$) in cows fed with CH and YEFECAP. Based on this study, it was concluded that providing CH or YEFECAP as protein source in concentrate diets could improve rumen fermentation and milk production in lactating dairy cows fed on rice straw.

Keywords: yeast-fermented cassava chips, cassava hay, rumen microorganism, milk production, lactating dairy cows

INTRODUCTION

The requirement for nutrients to support high milk production during early lactation is great. Cows in early lactation often suffer from a shortage of energy and protein because maximal DM intake does not occur until after the peak of milk production. Complex interrelationships exist between dietary protein, energy and the amount of protein that will be utilised by the dairy cow [1]. These interrelationships have important ramifications on overall N efficiency of the dairy farm. Dietary protein supplies metabolisable protein by providing both rumen degradable protein (RDP) utilised for microbial protein formation and rumen undegradable protein (RUP) that is digested directly by the cow.

The process of protein enrichment of animal feed using microorganisms in a semi-solid culture to improve the nutritional value of forage for ruminants has been evaluated [2-3]. Incorporation of microbial additives such as a culture of *Saccharomyces cerevisiae* to the diet has become common practice in ruminant nutrition. Boonnop et al. [4] reported that cassava chips fermented with *S. cerevisiae* (yeast-fermented cassava chips) significantly increase crude protein (300 g/kg DM) and lysine contents as well as reduce cyanide level.

Grown in tropical areas in large scale, Cassava (*Manihot esculenta*, Crantz) has a potential use in ruminant livestock nutrition and feeding. Cassava root contains a high level of energy and has been used as a source of readily fermentable energy in ruminant rations [5-7]. Whole cassava crop (cassava hay) was introduced by Wanapat [8] into a dry-season feeding system for ruminants by managing cassava crop growth in order to obtain optimal yield and good protein quality. Cassava hay is high in protein (200-250 g/kg DM) and contains condensed tannins (15-40 g/kg DM). It has proved to be an excellent ruminant protein feed and its use has been successfully implemented in several ways either by direct feeding or as a protein source in concentrated mixtures and high-quality feed blocks [8-9].

However, a comparative study of various protein sources in feed for ruminants has not yet been substantiated. It is therefore the objective of this investigation to determine the effects of yeast-fermented cassava chips, soybean meal, cassava hay and *Leucaena leucocephala* as protein sources in concentrated diets on feed intake, digestibility of nutrients, rumen fermentation, milk yield and milk composition of lactating crossbred dairy cows.

MATERIALS AND METHODS

Animals, Treatments and Experimental Design

Each of four crossbred (75% Holstein Friesian x 25% Thai native) early-lactating dairy cows with an average weight of 410±12.5 kg and 18±11 days in milk (DIM) was randomly assigned according to a 4×4 Latin square design to receive one of the four concentrated diets with different protein sources [soybean meal (SBM), cassava hay (CH), *Leucaena leucocephala* leaves (LL) and yeast-fermented cassava chips (YEFECAP)]. The composition of the feed concentrates is shown in Table 1. Cows were housed in individual pens and fed with the concentrated diets (ratio of concentrate to milk yield = 1: 2) twice daily at 6.00 a.m. and 16.00 p.m. after milking. All cows were additionally fed with urea-treated rice straw (UTRS) ad libitum as a roughage source while allowing for 10% refusal. UTRS (composition shown in Table 1) was made by pouring urea solution over a stack of straw

(urea : water : straw = 5 : 100 : 100 by weight), which was then covered with a plastic sheet for a minimum of 10 days before feeding directly to the animals [7].

Table 1. Ingredients and nutritional composition (g/kg DM basis) of feed concentrates (SBM, CH, LL and YEFECAP) and urea-treated rice straw (UTRS)

Ingredient (g/kg DM)	Protein source				UTRS
	SBM	CH	LL	YEFECAP	
Cassava chips	651	602	603	600	
Rice bran	80	80	76	67	
Molasses	20	19	19	16	
Soybean meal	189	-	-	-	
Cassava hay	-	231	-	-	
Leucaena leucocephala	-	-	237	-	
YEFECAP	-	-	-	255	
Urea	25	31	30	27	
Tallow	10	11	11	10	
Salt	10	10	10	10	
Mineral pre-mix	10	10	10	10	
Sulphur	5	5	5	5	
Nutritional composition					
Organic matter	938	945	929	941	905
Crude protein	183	181	180	182	79
Neutral detergent fibre	161	175	163	168	705
Acid detergent fibre	113	123	115	118	406

YEFECAP used in this study were described by Boonnop et al. [4]. In brief, cassava chips were washed and grated, and the processed pulp (100 g) was spread in a tray (about 50 cm diameter) to an average layer thickness of 2 cm. Commercial baker yeast (*Sacchromyces cerevisiae*, manufactured by Berly Speciality Industries Co., Bangkok) was used in the fermentation proceses. A nutrient solution was prepared by adding distilled water (100 mL), and then urea (48 g), to molasses (24 g) placed in a warm blender vessel flushed with O_2, and incubating the mixture at room temperature for 10 minutes. The resulting nutrient solution (250 mL) along with the yeast (20 g) was then inoculated into 0.5 kg of the processed pulp above and fermentation was conducted for 132 hours at 25°C. The fermented pulp was sun-dried for 3 days at an average temperature of 30°C and milled to give the YEFECAP (containing 300 g/kg DM).

All animals were kept in individual pens (4×6 m) and mineral block and water were freely available. The experiment was conducted in 4 periods according to 4x4 Latin square design (4 treatments and 4 periods), each period lasting 21 days. During the last 7 days of each period, samples were collected (diets, feces, milk, blood and rumen fluid).

Data Collection, Sampling Procedures and Methods of Analysis

Feed, refusal and fecal sample (grab sampling) were randomly collected (2 samples/day/cow) from each individual cow during the last 7 days of each period. Combined samples were dried at 60ºC and ground (1-mm screen, Cyclotech mill, Teactor, Sweden) and then analysed for DM, OM, ash, CP content [10], NDF, ADF [11] and acid-insoluble ash (AIA). The AIA was used to estimate digestibility of nutrients as described by Van Keulen and Young [12].

Cows were milked twice daily by a bucket-type milking system and milk was weighed at each milking of each period. Milk samples from both the morning and afternoon milking were combined daily, preserved with 2-bromo-2-nitropropane-1,3-diol and stored at 4°C until analysis of milk composition (fat, protein, lactose, total solids and solids-not-fat) by infrared method using Milko-Scan 33 (Foss Electric, Hillerod, Denmark). Milk urea nitrogen (MUN) was determined using Sigma kits #640 (Sigma Diagnostics, USA).

Rumen fluid was collected by a stomach tube connected with a vacuum pump and jugular blood samples were collected at 0 and 4 h post-feeding on the last day of each period. Approximately 200 mL of rumen fluid were taken from the rumen using a 60-mL hand syringe at the end of each period. The pH and temperature of the rumen fluid were immediately measured by means of a portable pH and temperature meter (Hanna HI 8424, Singapore). Rumen fluid samples were then filtered through two layers of cheesecloth and divided into three portions.

The first portion was used for analysis of volatile fatty acids (VFA) and NH_3-N. 1M H_2SO_4 solution (5 mL) was added to 45 mL of rumen fluid. The mixture was centrifuged at 16,000×*g* for 15 minutes and the supernatant was stored at -20°C prior to VFA analysis by HPLC (Waters, model 600E with a UV detector; Novapak C_{18} column, column size: 4 mm x 150 mm; mobile phase: 10 mM H_2SO_4, pH 2.5) according to Samuel et al. [13]. NH_3-N analysis was done by micro-Kjeldahl method [10].

The second portion was used for a total direct count of bacteria, protozoa and fungal zoospores with a haemacytometer (Hausser Scientific, USA) by the methods of Galyean [14]. The third portion was taken for the study of cultured groups of viable bacteria by roll-tube technique [15] for identifying rumen bacterial groups (cellulolytic, proteolytic, amylolytic and total viable bacteria).

A blood sample (about 10 mL) was drawn from the jugular vein at the same time as rumen fluid sampling (at 0 and 4 h post-feeding) and centrifuged at 5000×*g* for 10 minutes (Table-top Centrifuge PLC-02, USA). The supernatant was stored at -20ºC until analysis of blood urea nitrogen (BUN) according to the method of Crocker [16].

Statistical Analysis

Statistical analysis was performed using the GLM procedure of SAS (SAS Inst. Inc., USA). Data were analysed using the model $Y_{ijk} = \mu + M_i + A_j + P_k + \varepsilon_{ijk}$, where Y_{ijk} is observation from animal *j*, receiving diet *i* in period *k*; μ is the overall mean; *Mi* is the mean effect of protein sources (i = 1, 2, 3, 4); A_j is the effect of animal (j = 1, 2, 3, 4); P_k is the effect of period (k = 1, 2, 3, 4); and εijk is the residual effect. The results were presented as mean values and standard error of the means. Significant differences between treatments were determined by Duncan's new multiple range [17]. Differences among means with $P<0.05$ were accepted as statistically significant.

RESULTS AND DISCUSSION

Effect on the Rumen Ecology and Fermentation Products

The pattern of ruminal fermentation and overall means are presented in Table 2. Ruminal temperature and pH were similar among treatments and the values were quite stable at 39.1-39.4°C and pH 6.2-6.4, which was within the range (pH 6.0-7.0) considered for optimal microbial digestion of fibre and protein [7]. Ruminal NH_3-N, BUN and MUN ranged from 13.7-19.0, 11.3-15.7 and 13.5- 15.9 mg/dL respectively. Ruminal NH_3-N and BUN concentrations were lower in CH and YEFECAP than in SBM and LL. It was reported that ruminal NH_3-N concentration increased linearly with increasing supplemental RDP levels [6]. Therefore, a possible explanation for this could be that SBM and LL contain a high level of RDP, which leads to a high ruminal NH_3-N. Using the in sacco method, Promkot and Wanapat [5] found that effective degradability of CP in SBM and LL was higher than that found in CH. Wanapat [8] also reported that cattle fed on CH (250 g CP/kg) had lowered rumen NH_3-N and BUN concentration, which demonstrated the effect of condensed tannins in CH on the formation of tannin-protein complexes which in turn could enhance the cattle's rumen by-pass protein.

Table 2. Effect of protein source on some ruminal properties in lactating dairy cows (n=4)

Item	Protein source				SEM	P-value
	SBM	CH	LL	YEFECAP		
Ruminal pH	6.2	6.3	6.3	6.4	2.1	0.67
Ruminal temperature	39.2	39.3	39.1	39.4	1.1	1.02
NH_3-N, mg/dL	18.7^a	13.7^b	19.0^a	13.3^b	1.3	0.03
BUN, mg/dL	15.5^a	11.3^b	15.7^a	11.4^b	0.4	0.05
Total VFA, mmol/L	104.1^b	106.2^a	103.6^b	107.3^a	0.8	0.01
Mol % of total VFA						
Acetate (C2)	68.4	65.6	69.0	65.5	5.9	1.32
Propionate (C3)	23.6^b	25.4^a	23.2^b	26.5^a	0.2	0.02
Butyrate (C4)	8.0	9.2	7.8	8.0	2.5	2.22
Acetate to propionate ratio	2.9^a	2.5^b	3.0^a	2.4^b	0.1	0.05

Note: 1) ^{a,b,c} Means in the same row with different superscripts differ significantly ($P<0.05$).

2) SEM = Standard error of mean

The decreasing degradability of feed protein might also be due to an increase in the rumen outflow rate, thus lowering the time available for fermentation. Other authors found increased microbial N flow without changes in dietary N in the duodenum when yeast culture was added to the diet [18]. The other hypothesis could therefore be associated with yeast having a positive influence on ammonia uptake.

As NH_3-N is regarded as the most important nitrogen source for microbial protein synthesis in the rumen, the rumen pool of NH_3-N should be considered. The result obtained in this study was close

to optimal ruminal NH_3-N (15-30 mg/dL) [1-2, 6] for increasing microbial protein synthesis, feed digestibility and voluntary feed intake in ruminants fed on low-quality roughage.

The total VFA and propionic acid were significantly different ($P<0.05$) and were highest in CH and YEFECAP (Table 2). These values were similar to those reported by Wanapat et al [19]. The shift in the molar proportion of propionate resulted in a lower acetate:propionate ratio in ruminal fluid of animals receiving YEFECAP and CH. Wanapat et al. [19] reported that total VFA for CH supplementation increased with fermentation time in the rumen. However, recent data suggested that CH and YEFECAP improved rumen efficiency by increasing the C3 (propionate) intermediate and enhancing microbial protein synthesis in in vitro gas fermentation system [20].

Effect on Feed Intake and Digestibility

The effects of protein source on feed intake of lactating dairy cows are presented in Table 3. Dry matter intake (DMI) of UTRS and total DMI are shown to be similar. Normally, this data indicate that a source of protein has no negative effect on straw intake in dairy cows. This result is in agreement with earlier work by Khampa et al. [21], who reported that inclusion of cassava chips in diets resulted

Table 3. Effect of the main protein source in concentrated feed on voluntary feed intake and nutrient digestibility in lactating dairy cows (n=4)

Item	Protein source				SEM	P-value
	SBM	CH	LL	YEFECAP		
UTRS intake						
kg	5.8	6.0	5.7	6.1	1.9	0.43
g/kg BW	144	145	144	146	1.7	1.22
g/kg $BW^{0.75}$	65.6	66.5	64.9	67.0	2.8	0.67
Total feed intake						
kg	11.4	11.9	10.8	12.3	2.6	0.11
g/kg BW	290	293	288	293	2.4	0.23
g/kg $BW^{0.75}$	129.9	130.2	128.5	131.3	4.6	2.19
Apparent digestibility (g/kg DM)						
Dry matter	620	630	625	631	20.2	1.32
Organic matter	684	703	661	694	32.4	0.09
Crude protein	706 ^{b}	760^{a}	703 ^{b}	750^{a}	10.1	0.02
Neutral detergent fibre	614	632	593	643	25.3	0.55
Acid detergent fibre	562	581	553	584	17.6	0.28

Note: 1) ^{a,b} Means in the same row with different superscripts differ significantly ($P<0.05$).

2) g/kg $BW^{0.75}$ = gram / kilogram of metabolic weight; SEM = Standard error of mean

in satisfactory animal performance and had no negative effects on the health of lactating dairy cows. Apparent values of digestibility of DM, OM, NDF and ADF were not significantly different (P>0.05) among treatments. Wanapat et al. [19] also found that an increased ratio of CH to SBM in concentrate for dairy cows resulted in similar nutrient digestion coefficients among treatments. The CP digestibility values were significantly different and were highest in CH (760 g/kg DM) and YEFECAP (750 g/kg DM). Miller-Webster et al. [22] reported that protein digestibility and ammonia N were increased by inclusion of yeast culture as compared with control. This protein source could have made the N more available for microbial growth. Wanapat et al. [19] reported that both concentrate and CH were well consumed by cows at all times. However, Onwuka et al. [23] reported that dried cassava leaves contained high level of condensed tannins (30-50 g/kg DM), which adversely affected intake, digestibility and performance of ruminants.

Effect on Microbial Population

Table 4 illustrates data on rumen microbes using a direct count and roll-tube technique. Ruminal microbial count and cellulolytic and proteolytic bacteria were significantly different among treatments (P<0.05); bacteria, fungi zoospores, amylolytic bacteria and cellulolytic bacteria were highest when YEFECAP was supplemented. In contrast, the number of protozoa in the rumen was decreased by YEFECAP and CH supplementation. Although the effect of tannins on ruminal protozoa count is variable in assays carried out in vivo [20], some evidence exists for lower protozoal number in the presence of tannins [8-9]. Therefore, the decrease in protozoa count for CH supplementation could apparently be explained by the presence of condensed tannins in CH [8]. The effect of yeast culture on rumen protozoa is equivocal; whilst Robinson and Erasmus [24] reported that yeast culture exhibited no significant effect on the protozoa count, a trend for the total population to decrease in the presence

Table 4. Effect of the main protein source on microbial population in the rumen of lactating dairy cows (n=4)

Item	Protein source				SEM	P-value
	SBM	CH	LL	YEFECAP		
Total direct count (cells/mL)						
Bacteria, x 10^9	3.6^b	4.8^a	3.1^b	5.3^a	0.2	0.03
Protozoa, x 10^4	8.1^a	5.3^b	8.3^a	4.9^b	0.3	0.05
Fungi zoospores, x 10^3	2.8^b	3.9^{ab}	2.9^b	4.7^a	0.3	0.02
Roll-tube technique (CFU/mL)						
Total viable bacteria, x 10^8	4.8	5.1	4.9	5.2	2.9	1.12
Cellulolytic bacteria, x 10^7	5.2^c	6.0^b	5.1^c	7.5^a	0.2	0.04
Amylolytic bacteria, x 10^6	9.5	9.5	9.8	10.1	1.0	2.12
Proteolytic bacteria, x 10^6	11.0^b	12.1^{ab}	9.2^c	13.3^a	0.3	0.05

Note: 1) ^{a,b,c} Means in the same row with different superscripts differ significantly (P<0.05).

2) SEM = Standard error of mean

of *Saccharomyces cerevisiae* was observed [4,18]. Some authors reported elevation of total protozoa count when the animals were fed with low-quality diets, but the influence of *Saccharomyces cerevisiae* on the total population was much debated [25].

Guedes et al. [26] found that yeast could stimulate the activity of cellulolytic bacteria and increase lactate utilisation in the rumen, hence increased fibre digestion and flow of microbial protein from the rumen in feedlot cattle fed high-grain diets. Similarly, Erasmus et al. [18] reported that supplementation of yeast culture tended to increase microbial protein synthesis in dairy cows and significantly altered the amino acid profile of the duodenal digesta. When fungal cultures were supplemented in ruminant diets, it was found that microbial protein synthesis increased due to increase in microbial population in the rumen [27].

Effect on Milk Yield and Composition

The influences of protein source in concentrated diets on milk production and milk composition of lactating dairy cows are shown in Table 5. The protein source did not significantly affect milk yield, lactose, solids-not-fat and total solids ($P>0.05$). However, cows fed on CH or YEFECAP had higher milk fat than those supplemented with SBM or LL ($P<0.05$). A greater intake of urea-treated rice straw in the case of cows fed on CH and YEFECAP may partially explain our observed increase in milk fat. Dietary inclusion of yeast culture has shown an improved milk production in early-lactation dairy cattle [18, 24, 26]. All cows were able to maintain levels of milk yield during the days of the experiment. Similarly, Piva et al. [25] observed that milk fat increased significantly for mid-lactating cows fed diets with yeast in the concentrate. Wanapat et al. [19] reported that the fat content of milk was higher in CH-supplemented groups, especially in the ad libitum fed group. CH could have provided additional volatile fatty acids necessary for milk fat synthesis. Higher milk-fat percentage is good for milk price since the sale of milk is based on fat content.

Table 5. Effect of the main protein source on milk production and milk composition of lactating dairy cows (n=4, means of 7 days)

Item	Protein source				SEM	P-value
	SBM	CH	LL	YEFECAP		
Milk yield (kg/day)	15.0	15.6	14.7	15.7	2.2	0.98
Milk composition (g/100 kg of milk)						
Crude protein	3.1^{a}	3.3^{a}	2.2^{b}	3.3^{a}	0.1	0.03
Fat	3.7^{b}	3.8^{ab}	3.5^{c}	3.9^{a}	0.1	0.02
Lactose	4.9	5.0	4.8	5.1	1.2	0.05
Solids-not-fat	8.7	8.8	8.7	8.9	2.5	0.99
Total solid	12.7	12.8	12.5	12.9	1.8	1.22
MUN (mg/dL)	15.9^{a}	13.5^{b}	14.8^{ab}	13.9^{b}	0.3	0.05

Note: 1) ^{a,b,c} Means in the same row with different superscripts differ significantly ($P<0.05$).

2) SEM = Standard error of mean

Milk crude protein yield was greater in the CH- and YEFECAP-supplemented cows. The increased yield of milk crude protein may possibly be attributed to a greater passage of amino acids to the duodenum when CH or YEFECAP replaced SBM or LL in the diet. Higher ruminal by-pass protein (tannin-protein complex) of CH and higher amino acid content in YEFECAP could have contributed to this improvement [8]. In contrast, Kakengi et al. [28] showed that supplementation of LL to grazing cows significantly increased milk production, weight gain and milk composition, but had no significant effect on milk crude protein and solids-not-fat.

CONCLUSIONS

This study has revealed the importance of various protein sources for lactating dairy cows. Among the protein sources used, cassava hay (CH) and yeast-fermented cassava chips (YEFECAP) resulted in significantly higher rumen bacteria and fungal zoospore population as well as reduced protozoal population. The digestibility of protein also increased. Although milk yield was not different among treatments, milk protein and fat contents were enhanced in CH and YEFECAP supplemented cows. These protein sources could thus be recommended for use by smallholders.

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Communication

Microwave-assisted hydrothermal synthesis of lead zirconate fine powders

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Abstract: A rapid synthesis of lead zirconate fine powders by microwave-assisted hydrothermal technique is reported. The influences of type of lead precursor, concentration of potassium hydroxide mineraliser, applied microwave power and irradiation time are described. The synthesised powders were characterised by powder X-ray diffraction, field emission scanning electron microscopy, energy-dispersive X-ray spectroscopic microanalysis and light scattering technique. The merits of the microwave application in reducing reaction time and improving particle mono-dispersion and size uniformity as well as the drawbacks, viz. low purity of the desired phase and increasing demand of mineraliser, are discussed in relation to conventional heating method.

Keywords: lead zirconate, hydrothermal synthesis, microwave-assisted synthesis

INTRODUCTION

Lead zirconate ($PbZrO_3$ or PZ) is an important precursor in the preparation of a number of technologically important solid solutions, particularly those of the $PbZr_{1-x}Ti_xO_3$ series [1-2]. Recently, new studies have revealed novel applications of PZ as a phase-transformation-induced electro-mechanical actuator and a pyroelectric sensor [3-4]. The findings resulted in the reviving of interest in the synthesis of nano-sized PZ powders. Along this line, various synthetic techniques are available, e.g. hydrothermal, vibro-milling, sol-gel and precipitation, each of which exhibits characteristic drawbacks. The formation of aggregates, for instance, is an inherent problem for the hydrothermal technique [5-8]. The synthesis of fine PZ powders composing of mono-dispersed particles of uniform shape and size by this technique is therefore a challenge. According to our previous study, the fine powders of

orthorhombic PZ could be synthesised as a single phase under hydrothermal conditions at a temperature of at least 180°C for 24 hours [8]. The space group of the resulting PZ was the unusual *Pbma* with refined cell parameters a=5.88(6) Å and c=4.27(3) Å. The formation of aggregates was the major drawback. Nonetheless, the highly dispersed and well-defined cubic particles could be afforded when the reaction temperature and time were increased to 200°C and 72 hours respectively [8]. The attempt to use organic additives, e.g. polyvinyl alcohol, polyvinylpyrrolidone and cetyltrimethylammonium bromide, which are commonly employed in the synthesis of other oxide powders [9-11], may be an answer to this problem, although they need to be removed in the post-synthesis step.

Herein, the application of the microwave heating in the synthesis of PZ fine powders under hydrothermal conditions is reported. The primary objective of the synthesis is to promote the mono-dispersion of particles of the synthesised PZ powders without the assistance of organic additives. The influences of various synthetic parameters on the obtained powders and the advantages and shortcomings of the microwave heating are described and discussed.

MATERIALS AND METHODS

Equimolar mixtures of zirconyl nitrate hexahydrate [$ZrO(NO_3)_2.6H_2O$, 27% Zr (gravimetric), Fluka, 0.0115 mole] and one of the following lead precursors, i.e. lead(II) nitrate (99.0%, Univar), lead(II) acetate (99.5%, Ajax Chemicals), lead(II) fluoride (99.0%, Univar), lead(II) chloride (99.0%, Riedel de Haën) and lead(II) iodide (99.0%, Aldrich), were prepared in 10.0 cm^3 of deionised water. Pellets of potassium hydroxide (85%, Merck) was gradually added to each mixture with stirring to a concentration ranging from 6 to 14 moles dm^{-3}. Each mixture was then transferred to an 18-cm^3 Teflon reactor, which was sealed and placed in a 95(±5)°C water bath placed in a domestic microwave oven (Whirlpool XT-25ES/S, 900W, 2.45 GHz). The reaction was performed under autogenous pressure developed by the microwave heating (720W and 810W) for 3-5 hours. The resulting powder was recovered by filtration and washed with deionised water until the pH of the filtrate was approximately 7, followed a final washing with dilute acetic acid.

The synthesised powders were characterised for crystalline phases by powder X-ray diffraction (XRD) (using D8 Advance, Bruker, Cu $K\alpha$, Ni filter, λ=1.540598 Å, 40 kV, 30 mA). A field-emission scanning electron microscope equipped with energy-dispersive X-ray spectrometer (JSM-6335F, Jeol) was used in the examination of particle shape and size and elemental composition. In order to evaluate the aggregation, size distributions of the bulk powder samples were measured by light scattering technique (using Zetasizer Nano S, Malvern Instruments, 4mW He-Ne laser operated at 633 nm, particle size range 0.3nm-10μm).

RESULTS AND DISCUSSION

Although different lead precursors were used in the study, every reaction apparently provided the same off-white powders mixed with some red-block crystals, which were identified as lead oxide by XRD study. Although the contamination of lead oxide in the prepared powders seemed to be inevitable, they could be simply removed by washing with dilute acetic acid solution [12]. After the acid washing, it was found that only $Pb(NO_3)_2$ gave the well crystallised PZ while poorly crystallised powders were

obtained from the other lead precursors as depicted in Figure 1. This could be attributed to the low solubility of these lead precursors in aqueous solution compared to the readily dissolved $Pb(NO_3)_2$ [13]. The hardly dissolved lead precursors might bring about an insufficient nutrient in the solution and consequently the nucleation and crystal growth could not occur properly.

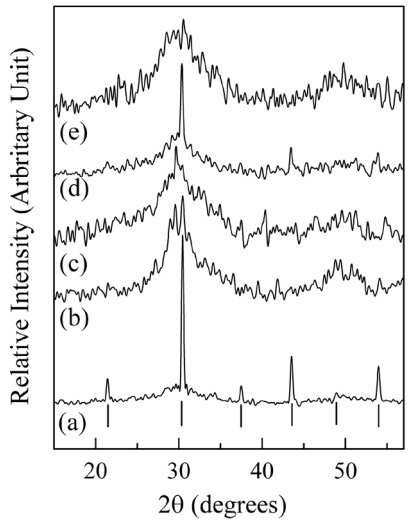

Figure 1. XRD patterns of the powders obtained from reactions between $ZrO(NO_3)_2.H_2O$ and different lead precursors in 14 moles dm^{-3} KOH solution for 3 hours: (a) $Pb(NO_3)_2$, (b) $Pb(CH_3COO)_2$, (c) PbF_2, (d) $PbCl_2$ and (e) PbI_2. The vertical bars indicate diffraction characteristics of PZ (ICSD 077-0856).

For the $Pb(NO_3)_2$ case, according to the XRD patterns of the acid-treated powders as shown in Figure 2, a very high KOH concentration of 14 moles dm^{-3} was required for the success of the synthesis. Lower concentrations resulted in poorly crystallised powders. However, a development in intensity of the *(200)* diffraction peak with increasing KOH concentration was apparent, suggesting the evolvement of the desired PZ. Another observation was a substantial reduction of the effective reaction time from days to hours when compared with the conventional hydrothermal synthesis [8].

According to former studies on similar issues, the observed phenomena could be explained as follows. Under commonly used hydrothermal conditions, the formation of nanocrystalline ZrO_2 as the hydrolysis product of aqueous $ZrO(NO_3)_2$ [14] could also be accelerated by microwave heating. This caused the breaking of large water clusters in the hydration sphere and the formation of the smaller ones [15]. Such mechanism increased the mobility of the dissolved lead species as well as the number of reaction sites on the occurring ZrO_2 surfaces for the hydrated lead ion-water clusters to react. A rapid synthesis should therefore be expected. According to the same studies, the hydrolysis of the aqueous $ZrO(NO_3)_2$ also resulted in the generation of nitric acid, which could neutralise the hydroxide species in solution [14]. In addition, there was evidence for the reduced electrolytic reaction of the KOH solution by the microwave radiation [16]. These phenomena were probably responsible for the increase in the high KOH concentration required in this study. This assumption was supported by the

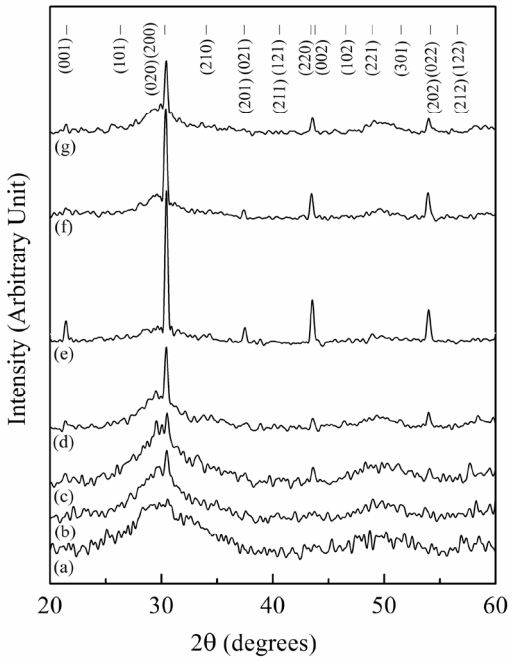

Figure 2. XRD patterns of the powders obtained from the reaction between $ZrO(NO_3)_2.H_2O$ and $Pb(NO_3)_2$ at 720 W for 3 hours in different concentrations of KOH solution: (a) 6 (b) 8 (c) 10 (d) 12 and (e) 14 moles dm^{-3}, compared to those obtained in 14 moles dm^{-3} KOH solution with different reaction times: (f) 4 hours and (g) 5 hours. The reference pattern is shown with vertical bars (ICSD 077-0856).

experiment in which a higher microwave power of 810 W was used. Rather than the desired PZ, mixtures of different oxides of lead and zirconium were obtained, which are the expected products of the hydrolysis reactions of the metal salts.

Based on the XRD patterns of the acid-treated powders, the synthesised PZ could be readily indexed as the orthorhombic *Pbma* phase with refined cell parameters a=5.87(1) Å, b=5.88(2) Å and c=4.15(2) Å (ICSD 077-0856). These results were well consistent with the conventional hydrothermal case, although the refined c parameter was shorter than the conventional heating case, c=4.27(3) Å [8], but closer to the standard, c=4.134 Å (ICSD 077-0856).

The field-emmision scanning electron microscopic (FESEM) images, as typically illustrated in Figure 3(a), showed that the powders largely consisted of discrete cubic particles although some particles with irregular shape were also present. The energy-dispersive X-ray spectroscopic measurements on the surface of several cubic particles indicated stoichiometric Pb:Zr, whereas the irregular-shape particles were found to be Zr-rich. This could account for the noisy background observed for the XRD patterns [Figure 2(e)]. The sizes of the cubic particles measured from the FESEM images were distributed in a significantly narrow range of 0.4-1.2 μm with approximately 60% of the population having a size of about 1 μm [Figure 4(a)]. Light scattering experiment on the bulk powder samples showed slightly larger particle sizes distributed mostly between 1-2 μm [Figure 4(b)]. The difference in particle sizes obtained from the two techniques should be due to a potentially biased

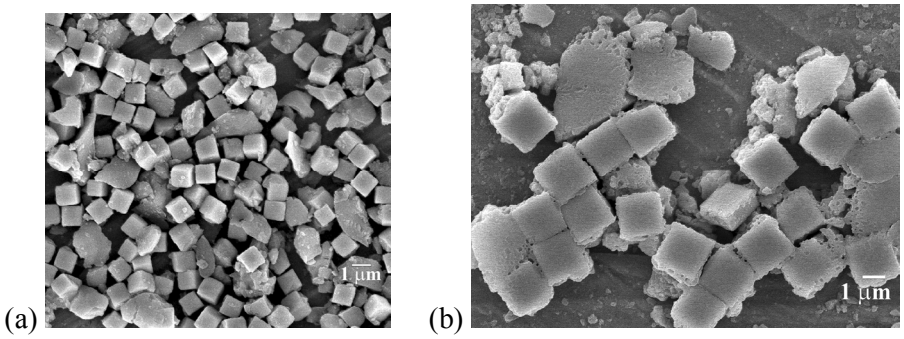

Figure 3. Typical FESEM photographs of PZ particles with corresponding XRD patterns shown in Figures 2(e) and 2(g) for crystals (a) and (b) respectively.

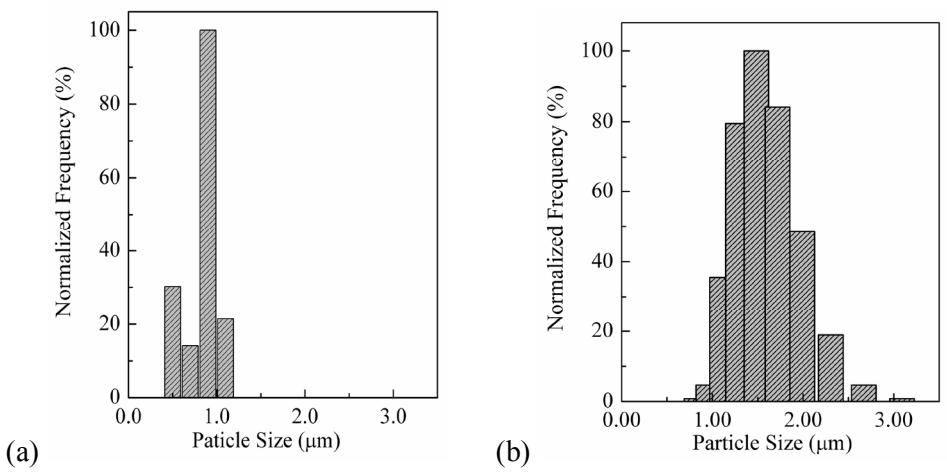

Figure 4. Particle size distribution of the cubic PZ particles measured from (a) FESEM images and (b) light scattering experiment (measured on the same powder sample)

analysis of the data. The non-spherical morphology of the particles normally introduces statistical errors to the laser light scattering data while a tendency of the particles to rest with preferred orientation on stub can induce bias in the data representation of the FESEM [17].

Thus, in comparison to the PZ powders derived from a conventional hydrothermal reaction where the particles were reportedly distributed in a 5-15 μm range with an average diameter of 7.5 μm [8], the pronouncedly narrower size distribution and the substantially smaller PZ particles have been achieved, although the negative effect on phase purity of the synthesised PZ and the contamination of some irregular Zr-rich particles were observed. A large number of nucleation induced by the localised microwave heating and the mechanism as described above, coupled with a poor crystal growth due to instantaneous and rapid ramping of such heating, could be the reasons for the approximate uniformity in the particle size [18-19]. Considering the PZ powders obtained from other chemical routes such as precipitation [20] and microemulsion [21], the apparent uniformity in particle shape and size may not be new. Both of these techniques can also give PZ powders composing of mono-dispersed spherical

particles typically 20 nm in diameter. However, calcination is required by both techniques, resulting in an unavoidable high temperature treatment and disadvantages incurred therein. As for the modified solid-state preparation of PZ powders, the problems with aggregation and particles with irregular shapes cannot be surmounted even though the vibro-milling is applied for over 25 hours [22].

The extension of the microwave heating time from 3 to 5 hours resulted in a reduction in intensity of the most intense *(200)* diffraction peak of the orthorhombic PZ and the appearing of a broad lump at the base of this peak as shown in Figure 2 (e-g). The FESEM images [Figure 3(b)] showed that the particles remained in the same cubic shape, although the bubbled surfaces and the necking between the adjacent particles could be observed. This indicated the boiling on the surfaces of these cubic PZ particles, which could be the result of a heat accumulation. The enlargement of the particles, which is a reflection of the particle growth process, and the disappearing of irregular particles with extended reaction time were also apparent.

CONCLUSIONS

In order to promote the mono-dispersion and the uniformity in shape and size of the hydrothermally derived PZ particles without the assistance of organic additives, microwave heating was attempted. The occurrence of lead oxide seemed to be inevitable and washing with dilute acetic acid was necessary. Among different variables studied, the type of lead precursor and the KOH concentration were most critical in the success of the synthesis. It was shown that the critical KOH concentration and the optimal microwave heating time were 14 moles dm^{-3} and 3 hours respectively. The highly dispersed cubic lead zirconate particles of approximately 1 μm in size could be synthesised in only 3 hour at 720 W of microwave heating. In comparison to conventional hydrothermal synthesis, the merits of the microwave method were clearly reflected by a shortening in hydrothermal reaction time, a reduction in particle size, an improved particle mono-dispersion, and a uniform particle size. However, the negative effects on the purity of synthesised powders, viz. the contamination of irregular particles, and the requirement for a very high concentration of potassium hydroxide mineraliser should be noted.

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Full Paper

Nutritional requirements for methyl orange decolourisation by freely suspended cells and growing cells of *Lactobacillus casei* TISTR 1500

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Abstract: *Lactobacillus casei* TISTR 1500 possesses cytoplasmic azoreductase and can breakdown azo bonds under microaerophilic condition. It was found previously that a growing culture is more tolerant to a high initial dye concentration than freely suspended cells supplied only with sucrose. The present study is aimed at investigating the nutritive requirements for decolourisation by the growing cells and the freely suspended cells using Plackett-Burmann experimental design. In this study, the composition of the medium was found to play an important role in methyl orange decolourisation and biomass production. Sucrose, meat extract and peptone increased methyl orange decolourisation by freely suspended cells, whereas sodium acetate exerted a negative effect on decolourisation. In addition, it was observed that the yeast and meat extracts enhanced the degradation of the dye by the growing cells. Sucrose was an important factor in biomass production by freely suspended cells and growing cells. On the other hand, dipotassium hydrogen phosphate and sodium acetate decreased the biomass production. These findings promote the understanding and knowledge about the requirements of azo dye decolourisation by *Lactobacillus casei*.

Keywords: *Lactobacillus casei*, microbial decolourisation, azo dyes, methyl orange, nutritional requirements

INTRODUCTION

Azo dyes are the most widely used synthetic colorants in comparison to natural dyes because of their many advantages, namely the ease and cost-effectiveness of synthesis, stability and availability in a variety of colours [1-2]. They are used in various industries such as pharmaceutical, food, brewing and cosmetic. However, several studies indicate that most of the azo dyes are toxic, carcinogenic and mutagenic [3-4]. Azo bonds are broken down by azoreductase to nitro-aromatic compounds [5]. Depending on the microorganisms, the biodegradation process can occur under aerobic or anaerobic conditions, or even a combination of the two. Aerobic microorganisms need to be acclimatised to produce a specific azoreductase to the dye whilst a universal azoreductase can be produced anaerobically without the process of acclimatisation [6]. In anaerobic condition, the mechanism starts with the reductive cleavage of the azo linkage and the reaction can occur in both intracellular or extracellular environments. The intracellular decolourisation requires a step of azo dye translocation from the environment into the bacterial cytoplasm [7]. However, the process of azo dye translocation across bacterial cell membrane is still under investigation. In a previous study, it was revealed that *Lactobacillus casei* TISTR 1500 requires sucrose or other types of sugars or organic acids as an energy source. The strain showed high potential of azo decolourisation by converting the dye to N, N-dimethyl-p-phenylenediamine and 4-aminobenzenesulphonic acid [5]. A few recent reports described the ability of lactic acid bacteria to degrade azo compounds. The microaerophile *Lactobacillus casei* LA 1133 degraded 35% of initial tartazine in 17 days with a growth rate at 0.052/h, and *L. paracasei* LA 0471 degraded 80% of tartazine in less than 13 days with a growth rate 0.023/h [8]. *L. acidophilus* ATCC 4356 completely reduced methyl red, orange G, Sudan III and Sudan IV while *L. fermentum* ATCC 23271 completely degraded only Sudan III and Sudan IV [9]. Also, *Oenococcus oeni* ML34 decolourised fast red up to 93% when the strain was supplied with 5 g/l of glucose [10].

Several factors such as dye structure, biomass concentration, alternative electron acceptor and redox mediator, dye concentration, and dye toxicity are known to influence the efficiency of decolourisation [11-12]. In another previous study, decolourisation by the strain TISTR 1500 of *L. casei* with a high cell density of freely suspended cells was examined to evaluate the performance of decolourisation as well as investigate the possibility of the process in azo dye treatment and factors affecting decolourisation (viz. sugars, organic acids, pH, temperature, oligosaccharides and metal ions) [7]. The nutritional requirements of the microorganisms are a key factor in biodegradation. The supplementation of wastewater contaminated with methyl red with phosphate, for example, improves decolourisation, whereas the addition of nitrate adversely affects organic reduction and decolourisation [13]. Also, yeast extract and peptone enhance decolourisation [14-15].

The strain TISTR 1500 had a high specific decolourisation rate of 14.2 mg/gCell/h and it was found that a growing culture is more tolerant to a high initial dye concentration than the freely suspended cells supplied only with sucrose [16]. Thus, in order to enhance the bacterial capacity for decolourisation, it is important to have some information related to the nutritive factors affecting the growth of the strain. The objective of the present study is to find the nutritional requirements of *Lactobacillus casei* TISTR 1500 for methyl orange decolourisation in a complex medium, particularly the MRS medium, and to create suitable culture media for growth and decolourisation.

The Plackett-Burman experimental design was applied to screening the main components of the MRS medium to establish a supportive condition for the growing cells and the freely suspended cells during methyl orange degradation. The information thus obtained should be important for an application of the process to the treatment and biodegradation of dyestuff wastewater.

MATERIALS AND METHODS

Chemicals and Equipment

Meat extract, peptone from casein and yeast extract were purchased from Difco, Dickinson and Co. (USA). Sucrose, dipotassium hydrogen phosphate, manganese sulphate, magnesium sulphate, sodium acetate and diammonium hydrogen citrate were purchased from Fisher Scientific. Tween 80 and methyl orange (C.I. 13025) were purchased from Sigma-Aldrich.

A Sorvall centrifuge (Super T21), a Genesys 10S UV-Vis Spectrophotometer (Thermo Scientific Inc.) and a Memmert incubator were used in the experiment.

Azo-dye-degrading Microorganism

Lactobacillus casei TISTR 1500 was obtained from a culture collection (MIRCEN part unit) of Thailand Institute of Scientific and Technological Research (TISTR). Subsequently, the strain was transferred from a lyophilised tube to 10 ml of modified MRS liquid medium [17] containing 0.5 g/l of methyl orange in a 20-ml screw-capped test tube and incubated at 35°C for 24 h. The cultivation was used as inoculum for the preparation of freely suspended cells.

Screening of Factors Affecting Microbial Growth

Plackett–Burman factorial design was employed for screening the basal medium components that support the growth of the strain TISTR1500 to decolourise methyl orange at 100 mg/l. Ten components based on the MRS medium were screened. Each factor was examined at two levels: -1 for low level and +1 for high level [18-19]. Table 1 depicts the Plackett–Burman experimental design with the ten factors under investigation and the levels of each factor used in the experimental design, which are based on the following first-order polynomial model (A):

$$Y = \beta_0 \sum \beta_i \chi_i \qquad \text{(A)}$$

where Y is the response (growth of microorganisms), β_0 is the model intercept, β_i is the linear coefficient, and x_i is the level of the independent variable.

This model was used for screening and evaluating important factors that influenced the response, although it did not describe the interaction among the factors. The positive or negative magnitude of the coefficient indicates the corresponding impact on titre. A coefficient value close to zero implies a small or no effect. The P-value is the probability describing the magnitude of a contrast coefficient that results from random process variability. A low P-value indicates a “real” or significant effect. The significance of each variable is determined by applying the F-ratio. In the present study, ten assigned variables (components) were screened in the course of twelve experimental runs. The experiments on the decolourisation rate and biomass were carried out in

triplicate. Based on the regression analysis of the variables, the confidence levels of 95% ($P<0.05$) and 90% ($P<0.1$) for each factor were considered to have a significant effect on the decolourisation and biomass production respectively.

Decolourisation of Methyl Orange and Analysis

The 12 runs of experimental media were set up as shown in Tables 1 and 2 and run under sterilised condition using aseptic technique. The culture of freely suspended cells and growing cells were prepared by the method of Seeuriyachan et al. [5, 16]. In the preparation of freely suspended cells, the strain was inoculated into 1 litre of modified MRS medium with 0.5 g/l of methyl orange in a 3-litre flask. It was then incubated at 35°C for 12 h, after which the cells were collected by centrifugation at 20,000×*g* for 10 min at 4°C. The pelleted cells were washed twice with 0.85% (w/v) NaCl and resuspended for further experiment with an initial OD_{600} of 0.3. For growing cells, the strain was inoculated into 10 ml of modified MRS medium containing 0.5 g/l of methyl orange in a 20-ml screw-capped test tube and incubated at 35°C for 24 h. It was then used as starter for further investigation and inoculated into 800 ml of the investigated medium (12 runs) in a 1-litre Erlenmeyer flask.

All treatments were incubated in a static condition at 35°C in an anaerobic jar and samples were collected every hour. All runs were performed in triplicate. The methyl orange concentration was measured spectrophotometrically from the supernatant at 444 nm and the decolourisation rate was determined using a curve plotting of dye concentration versus time. To determine the cell dry weight of the converted biomass, a standard curve was plotted between OD_{600} and cell dry weight (CDW) [16]. All determinations were done in triplicate.

Table 1. Assigned concentrations of variables at different levels in Plackett-Burman design for decolourisation of methyl orange by *Lactobacillus casei* TISTR 1500

Variable	Medium component	Lower level (-)	Higher level (+)
X1	Meat extract (g/l)	2	20
X2	Peptone from casein (g/l)	2	20
X3	Yeast extract (g/l)	1	10
X4	Sucrose (g/l)	4	40
X5	Dipotassium hydrogen phosphate (g/l)	0.4	4
X6	Manganese sulphate (g/l)	0.01	0.1
X7	Magnesium sulphate (g/l)	0.04	0.4
X8	Sodium acetate (g/l)	1	10
X9	Diammonium hydrogen citrate (g/l)	0.4	4
X10	Tween 80 (ml)	0.2	2

Table 2. Plackett-Burment design for 10 variables with 12 runs of experiment

Run no.	Component									
	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10
1	1	-1	1	-1	-1	-1	1	1	1	-1
2	1	1	-1	1	-1	-1	-1	1	1	1
3	-1	1	1	-1	1	-1	-1	-1	1	1
4	1	-1	1	1	-1	1	-1	-1	-1	1
5	1	1	-1	1	1	-1	1	-1	-1	-1
6	1	1	1	-1	1	1	-1	1	-1	-1
7	-1	1	1	1	-1	1	1	-1	1	-1
8	-1	-1	1	1	1	-1	1	1	-1	1
9	-1	-1	-1	1	1	1	-1	1	1	-1
10	1	-1	-1	-1	1	1	1	-1	1	1
11	-1	1	-1	-1	-1	1	1	1	-1	1
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1

RESULTS AND DISCUSSION

Nutritional Requirements for Decolourisation by Freely Suspended Cells and Growing Cells

A total of ten variables that influenced methyl orange decolourisation were analysed using the Plackett-Burman experimental design. Various medium components at different concentrations were investigated in the course of the study (Tables 1 and 2). The average decolourisation rates (observed values and predicted values) are shown in Table 3. The regression equations of the fitted model of the decolourisation and biomass production are represented in Table 4 and the predicted value of each response was generated therefrom.

To examine the fitting quality of the model, the values for each fitting method were compared. It was observed that the correlation coefficient (R^2) approaching 1 indicated a better fitting of the predicted values from the equations to the experimental values. The value of R^2 was 0.9945 for the decolourisation rate obtained using freely suspended cells, which could be interpreted as 99.45% variability in the response (Table 5). The magnitude and direction of the coefficient factor in equation (1) indicated the influence of the ten medium components on the decolourisation rate: a greater magnitude illustrated a larger effect. Variables with a confidence level greater than 95% ($P<0.05$) were considered significant.

It was found that with the response Y1 (decolourisation by freely suspended cells), four variables, namely sucrose (X4), meat extract (X1), sodium acetate (X8) and peptone (X2), had a low P-value of 0.0270, 0.0284, 0.0322 and 0.0334 respectively (Table 5). These variables thus significantly influenced the azo dye decolourisation. The estimated effects in the table indicate relative contribution of the variables on the response from the regression model. A positive value indicates that a higher setting of the variable resulted in a higher response while a negative value

Table 3. Comparison of methyl orange decolourisation rate between observed values and predicted values generated by the linear regression models

Run no.	Decolourisation rate by freely suspended cells (g/l/h)		Decolourisation rate by growing cells (g/l/h)	
	Observed rate	**Predicted rate**	**Observed rate**	**Predicted rate**
1	13.6	4.34	14.11	14.24
2	12.54	12.62	9.21	9.08
3	9.84	9.76	8.66	8.79
4	13.60	13.52	10.91	11.04
5	14.90	14.82	8.72	8.85
6	8.92	9.00	16.95	16.82
7	10.1	10.18	14.48	14.35
8	7.32	7.40	5.43	5.30
9	5.25	5.17	6.61	6.74
10	8.81	8.89	7.41	7.28
11	5.32	5.24	4.76	4.89
12	4.72	4.80	1.81	1.68

Table 4. Regression equations of the fitted models of decolourisation and biomass production by *Lactobacillus casei* TISTR 1500

Response	Equation
Y1 : Decolourisation by freely suspended cells	Y1 = 8.8117 + 1.7200X1+ 1.4583X2+0.2217X3+ 1.8067X4 + 0.3617X5 - 0.145X6 - 0.3333X7 - 1.5167X8 - 0.3183X9 + 0.7600X10.....(1)
Y2 : Decolourisation by growing cells	Y2 = 9.0883 + 2.1300X1 + 1.3750X2 + 2.6668X3 + 0.1383X4 - 0.1250X5 + 1.0983X6 + 0.0633X7 + 0.4233X8 + 0.9917X9 - 1.3583X10……(2)
Y3 : Biomass production by freely suspended cells	Y3 = 0.6075 + 0.0875X1 + 0.1025X2 + 0.1508X3 + 0.3192X4 - 0.2342X5 + 0.0758X6 - 0.0425X7 - 0.1575X8 + 0.1392X9 + 0.0692X10…….(3)
Y4 : Biomass production by growing cells	Y4 = 0.6225 + 0.0575X1 + 0.0842X2 + 0.0625X3 + 0.2625X4 - 0.1742X5 - 0.0058X6 - 0.0125X7 - 0.1642X8 + 0.0692X9 + 0.0208X10…….(4)

indicates the reverse effect (a lower setting resulting in a higher response). Sucrose had the highest estimated effect of 3.6133 on the decolourisation by the freely suspended cells of *L. casei* TISTR 1500. This implies that sucrose was an important factor in enhancing the decolourisation at a minimum concentration of 4 g/l. It thus follows that a deficiency of this component or using a lower sucrose concentration than this level could slow down the decolourisation. Sodium acetate, on the other hand, had a negative effect on the decolourisation. Addition of ammonium citrate or sodium acetate in the MRS medium is to inhibit other types of bacteria and fungal flora while favouring the growth of *Lactobacilli* [20]. For example, sodium acetate can stimulate the growth of *Lactobacillus salivarius* CRL 1328 and its bacteriocin production [21]. It also induces the production of lactic acid in *Lactobacillus sakei* NRIC 1071 and *Lactobacillus plantarum* NRIC 1067 [22].

Table 5. Estimated effect, linear regression coefficient of model, and the corresponding analysis of variance of F-ratio and P values for methyl orange decolourisation by freely suspended cells of *Lactobacillus casei* TISTR 1500 for the ten variables by Plackett-Burman experimental design at a confidence level of 95%

Component	Estimated effect	Coefficient	Standard error	Sum of squares	Df	Mean square	F-ratio	P- value
Intercept	8.8117	8.8117	0.0767					
X1	3.4400	1.7200	0.1533	35.5008	1	35.5008	503.3200	**0.0284**
X2	2.9167	1.4583	0.1533	25.5208	1	25.5208	361.8300	**0.0334**
X3	0.4433	0.2217	0.1533	0.5896	1	0.5896	8.3600	0.2120
X4	3.6133	1.8067	0.1533	39.1685	1	39.1685	555.3200	**0.0270**
X5	0.7233	0.3617	0.1533	1.5696	1	1.5696	22.2500	0.1330
X6	-0.2900	-0.1450	0.1533	0.2523	1	0.2523	3.5800	0.3096
X7	-0.6667	-0.3333	0.1533	1.3333	1	1.3333	18.9000	0.1439
X8	-3.0333	-1.5167	0.1533	27.6033	1	27.6033	391.3500	**0.0322**
X9	-0.6367	-0.3183	0.1533	1.2160	1	1.2160	17.2400	0.1505
X10	1.5200	0.7600	0.1533	6.9312	1	6.9312	98.2700	0.0640
Total error				0.0705	1	0.0705		
Total				139.7560	11			

Note: $R^2 = 0.9945$
Adjusted $R^2 = 0.9944$
Standard Error of Estimation = 0.2656
Mean absolute error = 0.0767

As indicated in Table 6, factors with P-value less than 0.05 were considered to have a significant effect on the response Y2 (decolourisation by growing cells). Only two variables [yeast extract (X3) and meat extract (X1)] with positive effects were selected as a source of nitrogen for the bacterial growth and decolourisation at 96.98% with a P-value of 0.0302, and at 96.22% with a P-value of 0.0378, with confidence levels at 1 and 2 g/l concentration respectively. It was evident

that yeast extract exercised the highest influence upon decolourisation by the growing cells with the highest estimated effect and regression coefficient of 5.3367 and 2.6683 respectively. Other factors proved to be statistically insignificant at a confidence level of 95%.

Table 6. Estimated effect, linear regression coefficient of model, and the corresponding analysis of variance of F-ratio and P values for methyl orange decolourisation by growing cells of *Lactobacillus casei* TISTR 1500 for the ten variables by Plackett-Burman experimental design at a confidence level of 95%

Component	Estimated effect	Coefficient	Standard error	Sum of squares	Df	Mean square	F-ratio	P-value
Intercept	9.0883	9.0883	0.1267					
X1	4.2600	2.1300	0.2533	54.4428	1	54.4428	282.77	**0.0378**
X2	2.7500	1.3750	0.2533	22.6875	1	22.6875	117.84	0.0585
X3	5.3367	2.6683	0.2533	85.4400	1	85.4400	443.77	**0.0302**
X4	0.2767	0.1383	0.2533	0.2296	1	0.2296	1.19	0.4720
X5	-0.2500	-0.1250	0.2533	0.1875	1	0.1875	0.97	0.5042
X6	2.1967	1.0983	0.2533	14.4760	1	14.4760	75.19	0.0731
X7	0.1267	0.0633	0.2533	0.0481	1	0.0481	0.25	0.7048
X8	0.8467	0.4233	0.2533	2.1505	1	2.1505	11.17	0.1851
X9	1.9833	0.9917	0.2533	11.8008	1	11.8008	61.29	0.0809
X10	-2.7167	-1.3583	0.2533	22.1408	1	22.1408	115.00	0.0592
Total error				0.1925	1	0.1925		
Total				213.7960	11			

Note: $R^2 = 0.9991$
Adjusted $R^2 = 0.9901$
Standard error of estimation = 0.4388
Mean absolute error = 0.1267

The results obtained indicated the difference in nutritional requirements between the freely suspended cells and the growing cells during process of decolourisation. The freely suspended cells of the strain TISTR 1500 required considerably more medium components than the growing cells. The strain required sucrose, meat extract and peptone to increase its decolourisation capacity. The growing cells could also increase the azo-dye decolourisation if adequate yeast extract and meat extract were supplied.

Nutritional Requirements for Biomass Production by Freely Suspended Cells and Growing Cells in the Presence of Methyl Orange

The main factors affecting biomass production were investigated by means of Plackett-Burmann experimental design using two types of cells of the strain TISTR 1500, namely the freely suspended cells at $OD_{600} = 0.3$ and the growing cells. The observed and predicted responses of the

Table 7. Comparison of biomass production during the process of decolourisation between freely suspended cells and growing cells at several media runs generated by Plackett-Burmann experimental design

Run no.	Biomass production by freely suspended cells (g/l)		Biomass production by growing cells (g/l)	
	Observed rate	Predicted rate	Observed rate	Predicted rate
1	0.42	0.45	0.43	0.45
2	1.25	1.22	1.10	1.08
3	0.52	0.55	0.53	0.55
4	1.47	1.50	1.20	1.22
5	0.53	0.56	0.84	0.86
6	0.18	0.15	0.16	0.14
7	1.62	1.59	1.36	1.34
8	0.34	0.31	0.43	0.41
9	0.35	0.38	0.38	0.40
10	0.32	0.29	0.35	0.33
11	0.16	0.19	0.25	0.27
12	0.13	0.10	0.44	0.42

12 different runs (Table 2) are given in Table 7. It can be seen that the observed biomass production by the freely suspended cells varied between 0.13-1.62 g/l, whereas the predicted values ranged between 0.1-1.59 g/l. The first-order model was generated using the experimental data. The screening of the MRS medium components was represented via the F-ratio for ANOVA. The estimated effect of the component variables on biomass production, the values of coefficients, the F-ratio and the P-value of each component from the response Y3 (biomass production by freely suspended cells) are represented in Table 8. From the design analysis of the regression coefficient, it was found that only sucrose showed a positive-effect value with an estimated effect of 0.6383, a coefficient of the regression model of 0.3192 and a P-value of 0.0646 on biomass production in the presence of methyl orange in the mixture. On the other hand, dipotassium hydrogen phosphate (X5) showed a negative effect with an estimated effect of -0.4683, a coefficient of the regression model of -0.2342, and a P-value of 0.0878 on the same. These components were screened based on their F-ratio and P-value at a confidence level of 90% ($P<0.1$).

As indicated in Table 9, three variables, namely sucrose (X4), dipotassium hydrogen phosphate (X5) and sodium acetate (X8) were the main factors influencing decolourisation by the growing cells of *L. casei* TISTR 1500. With the response Y4 (biomass production by growing cells; Table 4), sucrose enhanced the biomass production at a confidence level of 95% during the azo-dye decolourisation with an estimated effect of 0.5250, a coefficient of the regression model of 0.2625, and a P-value of 0.0424. In contrast, dipotassium hydrogen phosphate and sodium acetate showed a

Table 8. Estimated effect, linear regression coefficient of model, and the corresponding analysis of variance of F-ratio and P value for biomass production by freely suspended cells of *Lactobacillus casei* TISTR 1500 for the ten variables by Plackett-Burman experimental design at a confidence level of 90%

Component	Estimated effect	Coefficient	Standard error	Sum of squares	Df	Mean square	F-ratio	P- value
Intercept	0.6075	0.6075	0.0325					
X1	0.1750	0.0875	0.0650	0.0919	1	0.0919	7.25	0.2264
X2	0.2050	0.1025	0.0650	0.1261	1	0.1261	9.95	0.1955
X3	0.3017	0.1508	0.0650	0.2730	1	0.2730	21.54	0.1351
X4	0.6383	0.3192	0.0650	1.2224	1	1.2224	96.44	**0.0646**
X5	-0.4683	-0.2342	0.0650	0.6580	1	0.6580	51.91	**0.0878**
X6	0.1517	0.0758	0.0650	0.0690	1	0.0690	5.44	0.2578
X7	-0.0850	-0.0425	0.0650	0.0217	1	0.0217	1.71	0.4156
X8	-0.3150	-0.1575	0.0650	0.2977	1	0.2977	23.49	0.1295
X9	0.2783	0.1392	0.0650	0.2324	1	0.2324	18.34	0.1461
X10	0.1383	0.0692	0.0650	0.0574	1	0.0574	4.53	0.2796
Total error				0.0127	1	0.0127		
Total				3.0622	11			

Note: $R^2 = 0.9959$
Adjusted $R^2 = 0.9545$
Standard error of estimation = 0.1126
Mean absolute error = 0.0325

negative effect at a confidence level of 90% in biomass production with estimated effects of -0.3483 and -0.3283, coefficients of the regression model of -0.1742 and -0.1642, and P-values of 0.0638 and 0.0676 respectively. These results suggested that high concentrations of dipotassium hydrogen phosphate and sodium acetate at 4 g/l and 10 g/l decreased the biomass production, which was reflected by the decrease in decolourisation.

Lactic acid bacteria are fastidious microorganisms that require complex nutrients as they lack the ability to synthesise amino acids and vitamins. Thus, it is necessary to add complex nitrogen sources such as meat extract, peptone and yeast extract to their medium components. An interesting fact from this study is that sucrose is mainly required in the mixture with the freely suspended cells in order to increase the azo-dye decolourisation while meat and yeast extracts are the major stimulators of the decolourisation by the growing cells. In the case of *L. amylophilus* GV6, the strain does not require a high carbon source at 20 g/l of corn steep liquor for its growth and activity [23]. However, *Lactobacillus* sp. KCP01 requires a high carbon concentration at 25 g/l of reducing sugar for increasing the bacterial activity. In addition, all organic nitrogen sources (peptone, beef extract and yeast extract), dipotassium hydrogen phosphate and sodium acetate are positive factors for the strain [24-25]. Glucose is the main source of carbon for the growth of *Lactobacillus* sp. SK007 [26]. Under an acidic condition, glucose as a metabolisable carbohydrate like sucrose possesses

Table 9. Estimated effect, linear regression coefficient of model, and the corresponding analysis of variance of F-ratio and P values for biomass production by growing cells of *Lactobacillus casei* TISTR 1500 for the ten variables by Plackett-Burman experimental design at a confidence level of 90%

Component	Estimated effect	Coefficient	Standard error	Sum of squares	Df	Mean square	F-ratio	P- value
Intercept	0.6225	0.6225	0.0175					
X1	0.1150	0.0575	0.035	0.0397	1	0.0397	10.80	0.1881
X2	0.1683	0.0842	0.035	0.0850	1	0.0850	23.13	0.1305
X3	0.1250	0.0625	0.035	0.0469	1	0.0469	12.76	0.1738
X4	0.5250	0.2625	0.035	0.8269	1	0.8269	225.00	**0.0424**
X5	-0.3483	-0.1742	0.035	0.3640	1	0.3640	99.05	**0.0638**
X6	-0.0117	-0.0058	0.035	0.0004	1	0.0004	0.11	0.7952
X7	-0.0250	-0.0125	0.035	0.0019	1	0.0019	0.51	0.6051
X8	-0.3283	-0.1642	0.035	0.3234	1	0.3234	88.00	**0.0676**
X9	0.1383	0.0692	0.035	0.0574	1	0.0574	15.62	0.1578
X10	0.0417	0.0208	0.035	0.0052	1	0.0052	1.42	0.4448
Total error				0.0037	1	0.0037		
Total				1.7544	11			

Note: $R^2 = 0.9979$
Adjusted $R^2 = 0.9769$
Standard error of estimation = 0.0606
Mean absolute error = 0.0175

protective effects as bacteria demand high energy for maintaining pH homeostasis [27-28].

Peptone is a rich source of amino acids and partially involves pH homeostasis mechanism. Increasing the peptone concentration in the medium can enhance the buffering capacity and bacterial survival [29]. In this case of *Lactobacillus casei* TISTR 1500, the increasing rate of azo dye degradation might have caused the buffering effect when the medium contained high peptone concentration. Both peptone and yeast extract have a positive effect on the growth of *L. fermentum* [30] and *L. lactis* subsp. *lactis* [31]. As yeast extract is a rich source of amino acids and vitamins, this could account for its positive influence on the bacterial growth [25, 32]. Attempts have been made to replace yeast extract in stimulating the bacterial growth and lactic acid production by various nitrogen sources. However, none of these sources are comparable to yeast extract, nor do they yield bacterial productivity as high as yeast extract [33].

In our previous study [5, 16], pH was seen as a limiting factor of dye decolourisation for the strain TISTR 1500. Besides, meat extract and peptone were observed to play an important role in controlling the pH [34]. For the strain TISTR 15000, the mechanism of methyl orange decolourisation starts with azo dye translocation across the bacterial membrane [16]. A low pH with a high concentration of lactic acid can lead to the disruption of some metabolic pathways. Thus, a high buffering capacity of the mixture can increase the decolourisation rate as demonstrated in the

present study, the results of which have also shown the unconventional findings on the difference in requirements between the freely suspended cells and the growing cells. These finding suggest that during decolourisation the freely suspended cells require a high buffer capacity or pH regulation in comparison to the growing cells.

The results obtained in the present study for the strain TISTR 1500, with sucrose being the only main positive factor and the inorganic phosphate and sodium acetate exerting a negative influence on biomass production in the presence of an azo dye, apparently differ from other studies. It was observed that lactose and peptone have a positive influence on biomass production [35]. Bevilacqua et al. demonstrated an increasing trend in the biomass production when the carbon source increased up to 20 g/l in a nonlinear way at pH 6, but the interaction effect between carbon source and pH on biomass production was small [36].

As mentioned in our previous study [16], the strain TISTR 1500 possesses cytoplasmic azoreductase and the azo dye has to be imported through the bacterial membrane in the first step of the dye degradation. The study of Schär-zammaretti et al. [37] suggests that the morphology and structure of the bacterial cell wall changes depending on the composition of the medium. Both peptone and yeast extract have the major influences on the physicochemical properties of the cell wall, particularly the membrane-bound proteins. They may cause a change in the hydrophobicity of the cell wall. In MRS medium, the cell wall has a low hydrophobicity in the absence of carbohydrates. However, in the absence of peptone and yeast extract in the MRS medium, the hydrophobicity of the bacterial cell wall becomes high. Similarly, the electrical charge on the bacterial cell wall surface correlates with its N/C ratio [37]. A change in the cell wall structure and its physicochemical properties can thus affect the rate of azo dye translocation across the cell membrane. The current findings of this study should be useful for improving the culture media for the strain TISTR 1500 in order to rejuvenate the microbial cells when the strain is applied in a system of wastewater treatment.

CONCLUSIONS

The composition of the fermentation medium has been observed to be a major factor affecting the methyl orange decolourisation capacity and biomass production of the strain TISTR 1500 of *Lactobacillus casei*. A difference in the nutritional requirements of the freely suspended cells in comparison to the growing cells has also been demonstrated. Sucrose, meat extract and peptone increased methyl orange decolourisation by the freely suspended cells while sodium acetate had a negative effect on the decolourisation. Both yeast extract and meat extract enhanced the degradation of the azo dye by the growing cells. Sucrose was found to be important for the biomass production by freely suspended cells and growing cells in the presence of 100 mg/l of methyl orange. On the other hand, dipotassium hydrogen phosphate and sodium acetate decreased biomass production. These findings should promote an understanding of the requirements of azo dye decolourisation by *Lactobacillus casei*.

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Full Paper

Hybrid QM/MM study on the deglycosylation step of chitin hydrolysis catalysed by chitinase B from *Serratia marcescens*

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Abstract: Chitinase B (ChiB) from *Serratia marcescens* is an exo-chitinase that degrades chitin chains from the non-reducing end by cleaving off a dimer or trimer sugar product. This enzyme features the substrate-assisted mechanism for the cleavage of the β-1,4-glycosidic bond. In this study, the deglycosylation step of chitin hydrolysis catalysed by *S. marcescens* ChiB is investigated by hybrid QM/MM method. Potential energy surface calculated at AM1/CHARMM22 level shows the presence of two transition states (TS1 and TS2) indicating that the deglycosylation path follows a stepwise mechanism in which the nucleophilic attack of catalytic water at the anomeric carbon occurs as the first step followed by proton abstraction. The calculated potential energy barriers for TS1 and TS2 are 26.8 and 4.0 kcal/mol, suggesting that the nucleophilic attack by water molecule is the rate-limiting step for the deglycosylation. In addition, an oxocarbenium-like character in TS1 can also be captured.

Keywords: chitinase B, *Serratia marcescens*, QM/MM, enzyme catalysis, glycoside hydrolase

INTRODUCTION

Chitinase B (ChiB) (EC 3.2.1.14) belonging to a member of glycoside hydrolase (GH) family 18 [1-2] effects the hydrolysis of chitin, a linear insoluble polymer of N-acetylglucosamine (GlcNAc or NAG), which is believed to be the second most abundant polysaccharide on earth after cellulose. This enzyme has received much attention as an attractive target for development of new drugs/inhibitors [3] with chemotherapeutic potential against fungi, insects and malaria transmission or anti-inflammatory potential against asthma and allergic diseases. Designing such inhibitors with sufficiently high selectivity and affinity requires a detailed understanding of the catalytic mechanism at the molecular level.

Catalysis by ChiB from *Serratia marcescens* has been proposed to involve a substrate-assisted double displacement which leads to the retention of configuration at the anomeric carbon [4-6]. The overall reaction of ChiB proceeds in two main steps, i.e. glycosylation and deglycosylation. Two key catalytic residues (Asp142 and Glu144) were identified previously by both structural and kinetic studies to play an important role in catalysis by ChiB [4, 6-7]. In the glycosylation step, Asp142 assists in the formation of an oxazolinium ion intermediate by polarising the 2-acetamido group of the substrate to increase its nucleophilicity, thereby promoting attack of the carbonyl oxygen (O7) at the anomeric centre (C1). Glu144 meanwhile acts as a general acid, encouraging departure of the aglycon leaving group [8] by means of proton transfer from Glu144. In the deglycosylation step (Figure 1), the oxazolinium intermediate is broken down in a reverse manner to the glycosylation step: Glu144 is believed to act as a general base, promoting attack of a water molecule at the anomeric center (C1), while Asp142 is thought to facilitate the expulsion of the 2-acetamido group from the anomeric centre (C1), yielding a sugar hemiacetal product with overall retention of stereochemistry.

Hybrid or combined QM/MM (quantum mechanics/molecular mechanics) approach, introduced to enzyme reactions by Warshel and Levitt [9], treats the central reacting system with a quantum chemical method while representing the remainder of the system by a classical empirical force field model. This technique has an advantage not only of verifying a proposed reaction mechanism, but also of elucidating a transition state or intermediate, a performance that may not be achievable by experimental methods. Application of this technique to the study of an enzymatic reaction has increased significantly over the past two decades [10-11]. For the GH enzymes, this QM/MM technique has increasingly been applied and has provided new insights into the hydrolysis of glycosidic bonds, as evidenced by recent QM/MM studies on various enzymes such as lysozyme [12], endoglucanase [13], β-galactosidase [14], cellulase [15] and Golgi α-mannosidase [16]. However, thus far no work has been reported on the QM/MM study of the ChiB-assisted hydrolysis mechanism. Very recently, our group [17] has studied the glycosylation step by *S. marcescens* ChiB by using the combined QM/MM method. Here, we aim to continue our previous QM/MM study in order to fully understand the overall action of ChiB.

In this study, the hydrolysis of oxazolinium ion intermediate (deglycosylation step) by *S. marcescens* ChiB is investigated in detail by using hybrid QM/MM calculations. The QM/MM potential energy surface calculated at AM1/CHARMM22 level is carried out to track a possible reaction pathway and locate and characterise the structures corresponding to the stationary points in

the deglycosylation path, thus giving important insights into the mechanism of deglycosylation reaction of chitin catalysed by *S. marcescens* ChiB.

METHODS

Model Preparation

The starting model of the ChiB-intermediate complex in the deglycosylation step was built on the basis of X-ray crystallographic structure PDB codes [6] of 1E6R and 1E6Z for the *S. marcescens* ChiB complexed with the allosamidin inhibitor and its reaction intermediate respectively. These two crystal structures were superimposed by using chain A of 1E6Z as a template to generate the ChiB-intermediate model structure containing a dimeric product (NAG_2 at subsite –1, –2) and a leaving group of dimeric sugar (NAG_2 at subsite +1, +2) as shown in Figure 4. These subsite numbers indicate the sugar binding subsite of NAG_5 (–2 to +3) [6]. The observed catalytic water molecule and other crystallographic water molecules were kept. Hydrogen atoms were added using HBUILD module in CHARMM [18] with all residues in their physiological protonation state. The only exception was the acid/base residue (Glu144), which was deprotonated in the second step as proposed by van Aalten et al. [6] while the Asp142 residue remained protonated.

QM/MM MD Simulation

In QM/MM MD simulation, the system was partitioned into QM and MM regions. The QM region consisted of the putative oxazolinium ion intermediate, one catalytic water molecule and side-chain groups of Asp142 and Glu144, leading to a total of 46 atoms (Figure 1). These QM residues were treated with AM1 semi-empirical method [19], which has been shown to satisfactorily describe the first step of ChiB-assisted reaction [17]. Three bonds crossing the QM/MM boundary were capped with the 'QQ link atoms' (Figure 1). The charge on the QM system was neutral. Other protein (5871), water (1650) and sugar atoms (131) were treated using the modified version of all-atom CHARMM22 MM force field [20]. The simulations employed a stochastic boundary approach [21]. The system was solvated by superimposing a 25-Å-radius sphere of TIP3P water molecules centred on the anomeric C1 carbon and equilibrated for 1.6 ns of CHARMM QM/MM molecular dynamics (MD) by a procedure similar to that of Lodola et al. [22]. MD snapshots were chosen for subsequent restrained QM/MM minimisation with adopted-basis Newton-Raphson method until the gradient was less than 0.01 kcal/(mol•Å). The resulting minimised structures were selected as starting point for reaction path calculation. The simulation was carried out with CHARMM version 27b2 [18].

Reaction Path Calculation

The two-dimensional (2D) potential energy surface (PES) of a reaction path was determined by adiabatic mapping calculation using CHARMM's RESDistance facility [18]. The reaction was described by two discrete coordinates (R_X and R_Y) to represent the two key individual steps of the deglycosylation. As illustrated in Figure 1, a proton (H_w) is abstracted from the catalytic water molecule by Glu144 (event ***Y***), which is described by $R_Y = r[O_w,H_w] - r[O\varepsilon_1,H_w]$. After that the

deprotonated water attacks the anomeric C1 carbon, resulting in a collapse of the oxazolinium ion intermediate (event ***X***), which is described by $R_X = r[C1,O7] - r[C1,O_w]$. R_X and R_Y were increased in steps of 0.2 Å with harmonic restraints of 5000 kcal/mol•Å^2. Geometrical optimisation of the structures was performed at each point until the gradient was less than 0.01 kcal/(mol•Å). The energy was then computed by a single-point calculation, which removed the energy contribution due to reaction coordinate restraints.

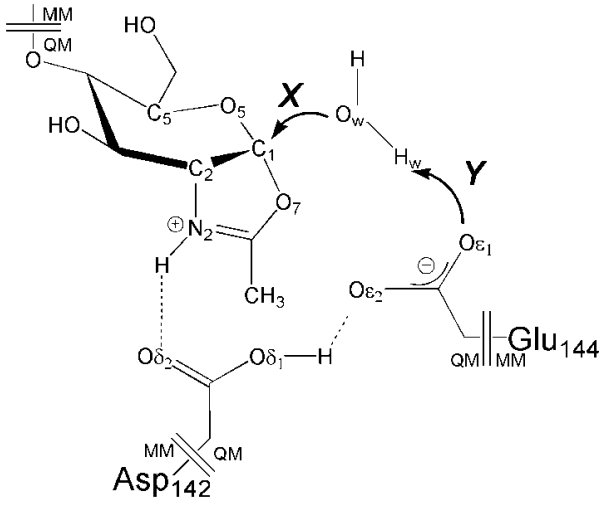

Figure 1. QM regions used in this study showing two individual steps for the deglycosylation by *S. marcescens* ChiB (***X*** and ***Y*** represent the nucleophilic attack of catalytic water and proton abstraction respectively)

RESULTS AND DISCUSSION

Structure of ChiB-Intermediate Complex

The model of ChiB-intermediate complex is quite stable during the QM/MM MD simulation, as demonstrated by the root mean square deviation (RMSD) of about 0.44 ± 0.07 Å in Figure 2. Table 1 lists some important geometric parameters from the X-ray experiment, QM/MM MD simulation and QM/MM minimisation. As can be seen, the geometry from our simulation is in good agreement with the X-ray structure. Tyr214 and Asp142 show very stable H-bonds (s.d. = 0.22 and 0.11 Å respectively) during the QM/MM MD simulation (Table 1), indicating the importance of these two residues in stabilising the intermediate (oxazolinium ion). Importantly, the torsional angle (C2–C1–O5–C5) representing the chair conformation of the oxazolinium ion ring is very stable during the simulation, which is in excellent agreement with the X-ray experiment, indicating the suitability of the AM1 method in representing the conformation of the putative oxazolinium ion ring. Although the simulated distances of C1–O_w and Asp142:Oδ1–Oε2:Glu144 seem to be greater than those from experiment, they were found to improve during the restrained QM/MM minimisation as shown in Table 1, giving a reasonable starting point for subsequent reaction path calculation.

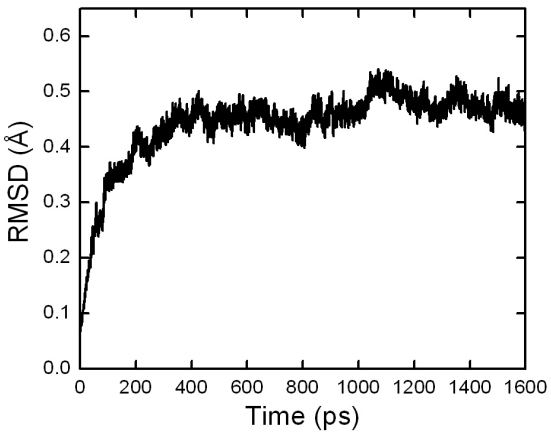

Figure 2. Root mean square displacement (RMSD) of the heavy atoms of ChiB-intermediate complex during 1.6 ns of QM/MM MD simulation

Table 1. Some important geometric parameters obtained from X-ray experiment, simulation (QM/MM MD) and minimisation (QM/MM)

Geometric parameter [a,b]	Experiment [c]	Simulation	Minimisation
C1–O_w	2.75	3.40 ± 0.50	2.76
Glu144:$O\varepsilon_1$–O_w	2.67	2.96 ± 0.51	2.54
Asp142:$O\delta_1$–$O\varepsilon_2$:Glu144	2.26	3.70 ± 0.55	3.04
N2–$O\delta_2$:Asp142	2.92	2.88 ± 0.11	2.78
Tyr214: OH–O7	3.05	3.10 ± 0.22	3.12
O_w–C1–O7	152.6	165.8 ± 8.8	146.2
C2–C1–O5–C5	–43.5	–43.5 ± 6.1	–44.2

[a]Atomic numbering corresponds to Figure 1, whereas other atom types follow the CHARMM format.

[b] Distances are given in angstroms; angles (bond angle and torsional angle) are given in degrees.

[c] from X-ray crystal structure (PDB code 1E6Z) [6]

Reaction Path

Figure 3 shows the AM1/CHARMM22 potential energy surface (PES) describing two key events (***X*** and ***Y*** in Figure 1): the nucleophilic attack of oxygen (O_w) of catalytic water at the C1 anomeric carbon (R_X) and the proton (H_w) abstraction from water molecule by oxygen atom ($O\varepsilon_1$) of the deprotonating Glu144 (R_Y). The stationary points for minima and saddle points located on the PES are also depicted.

As indicated in Figure 3, the PES clearly shows the presence of two transition states (TS1 and TS2) indicating a stepwise mechanism in which the nucleophilic attack occurs as the first step followed by the proton abstraction. This observation is unexpected as the deglycosylation reaction

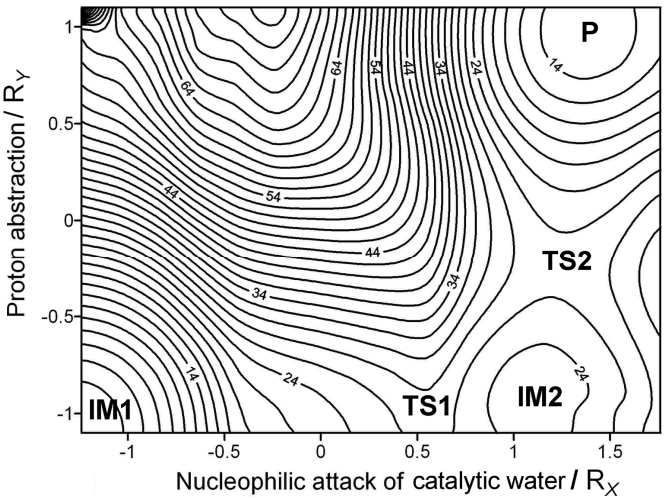

Figure 3. AM1/CHARMM22 potential energy surface for the deglycosylation reaction catalysed by *S. marcescens* ChiB [IM1 = oxazolinium intermediate 1; TS1 = transition state 1; IM2 = intermediate 2; TS2 = transition state 2; P = dimer product (NAG_2)]. The unit of QM/MM energies is in kcal/mol.

has been proposed to occur via only one transition state as well as the glycosylation [6]. This findings have suggested a new mechanism for the deglycosylation by *S. marcescens* ChiB. The changes of the QM/MM energy for IM1 → TS1 and IM2 → TS2 are 26.8 and 4.0 kcal/mol respectively (Table 2), indicating that the rate-limiting step for the deglycosylation reaction is the nucleophilic attack by catalytic water. The calculated barrier of about 27 kcal/mol is considerably higher than the experimentally determined barrier of 16.1 kcal/mol estimated from the experimental rate constant of 40.9 s^{-1} at 310 K [23] using transition state theory [24]. It should be noted, however, that such experimental barrier was estimated based on the overall reaction (i.e. glycosylation and deglycosylation). Therefore, although a high value of calculated barrier is observed in this study, an accurate energy barrier is not expected. More importantly, as no clear evidence on the presence of a rate-determining step in ChiB-assisted reactions has been reported previously, new insight into the catalytic mechanism of the deglycosylation by *S. marcescens* ChiB at molecular level has thus been gained.

The stabilisation energy of the reactive site (QM region) provided by the protein environment (MM region) along the stationary points has also been analysed relative to the energy in IM1. The result indicates that the influence of the protein environment on the stabilisation of the reactive site gradually decreases, as reflected by the negative values of stabilisation energy (Table 2), which provides evidence that the intermediate state (IM1) is central to the catalysis by ChiB.

Geometry along the Reaction Path

Snapshots of stationary structures (IM1, TS1, IM2, TS2, and P) and their corresponding geometric parameters along the deglycosylation path are shown in Figure 4 and Table 2 respectively. The starting structure of the ground state of ChiB-intermediate (IM1) indicates a suitable

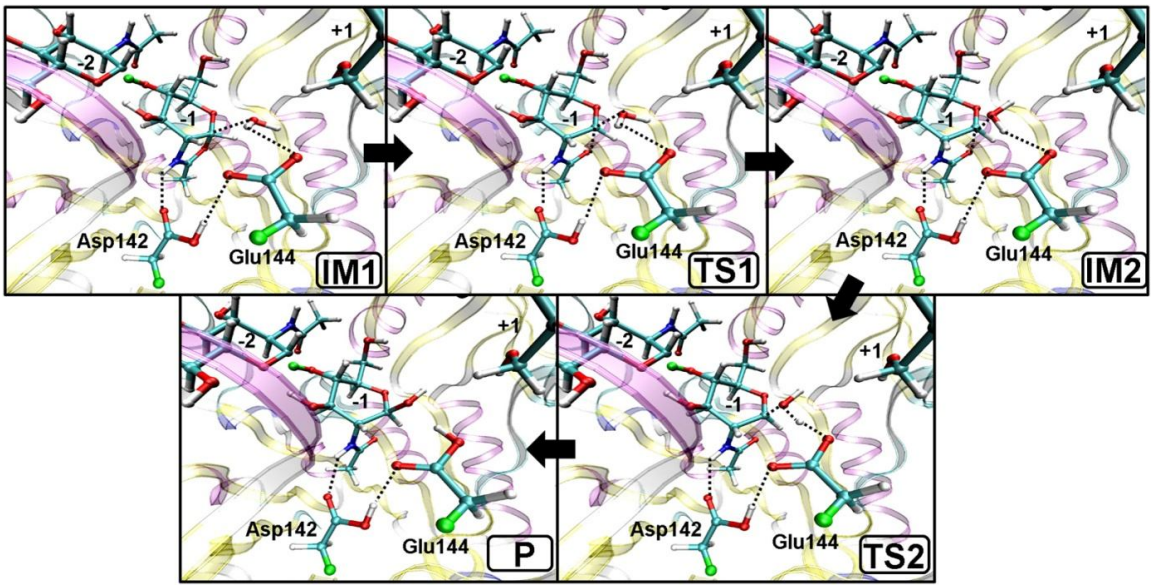

Figure 4. Snapshots of the stationary structures along the deglycosylation path

Table 2. Relevant geometric parameters and energy values of different stationary structures on the PES calculated at AM1/CHARMM22 level

Parameter [a]	IM1	TS1	IM2	TS2	P
C1–O_w	2.73	1.96	1.53	1.50	1.43
C1–O7	1.49	2.52	2.69	2.66	2.79
C1–O5	1.39	1.32	1.38	1.39	1.41
O_w–H_w	0.97	0.98	1.02	1.15	2.00
Glu144:Oε_1–H_w	2.07(2.56)[b]	2.09	1.92	1.35	1.00
Asp142:Hδ_1–Oε_2:Glu144	2.70(3.64)[b]	2.82	2.78	2.29	2.48
HN2–Oδ_2:Asp142	2.09(2.83)[b]	2.13	2.13	2.13	2.21
O_w–C1–O7	147.7	150.8	159.1	156.6	151.8
C2–C1–O5–C5	–27.8	–11.1	5.1	1.6	0.4
QM/MM energy	0	26.8	22.7	26.7	12.3
Stabilisation energy [c]	0	–22.6	–27.1	–48.9	–71.4

[a] Distances are given in angstroms; angles (bond angle and torsional angle) are given in degrees; and energies are given in kcal/mol.
[b] Heavy atom distance
[c] Stabilisation energy is the energy difference between full QM/MM system and only QM atoms relative to that of IM1.

position for nucleophilic attack of water molecule as evidenced by the distances of C1–O_w (2.73 Å) and Glu144:Oε_1–H_w (2.07 Å) and the O_w–C1–O7 angle (147.7º), which is in good accordance with the X-ray structure shown in Table 1. It is also observed from the X-ray experiment [6] that the chair conformation of the oxazolinium ring is distorted by only about 15º, indicating a relaxed conformation of the ring in the beginning of the nucleophilic reaction.

In TS1, the water molecule makes an attack at the anomeric centre (C1) to some extent (Table 2), which is close enough to break the oxazolinium ring by lengthening the C1-O7 distance ~1.0 Å from the IM1. At the same time, the chair conformation of the oxazolinium ring is distorted to a half-chair, as shown by a more planar conformation around the C1–O5 bond (C2–C1–O5–C5 = −11º). Such planarity, together with a shortening C1–O5 bond to a partial double bond (1.39 Å at IM1 to 1.32 Å at TS1), indicates the formation of an oxocarbenium-like ion. Note that this half-chair conformation is found to remain until the product state but with increasing trend of planarity (see C2–C1–O5–C5 in Table 2). The Glu144:Oε_1–H_w distance does not change much at this stage, suggesting that no proton abstraction occurs here.

In IM2, as can be seen in Figure 4, the water molecule orients itself to be poised for in-line attack at the anomeric C1 atom as shown by the largest O_w–C1–O7 angle (~160º) and to facilitate the proton abstraction, which initially happens in this stage, as indicated by a slight change in the O_w–H_w distance (0.98 Å at TS1 to 1.02 Å at IM2) and the Glu144:Oε_1–H_w distance (2.09 Å at TS1 to 1.92 Å at IM2). Meanwhile, the oxygen atom of catalytic water moves closer to the anomeric centre (1.96 Å in TS1 to 1.53 Å in IM2) and again the increasing C1–O7 distance (2.52 Å in TS1 to 2.69 Å in IM2) is also observed.

In TS2, the proton abstraction from the water molecule to Glu144 is almost complete at this stage (Figure 4): the distance of the moving proton to the oxygen of Glu144 (Oε_1–H_w) is 1.15 Å, whereas the distance to the water oxygen (O_w–H_w) is 1.35 Å. During the proton abstraction, Glu144 is stabilised by the hydrogen atom from Asp142 as indicated by a large decrease in the Hδ□–Oε2 distance (2.78 Å at IM2 to 2.29 Å at TS2).

In the product (P), the proton abstraction is complete after Glu144 accepts the hydrogen atom (H_w) from the deprotonated water, resulting in the expulsion of the 2-acetamido group from the anomeric C1 carbon (2.66 Å at TS2 to 2.79 Å at P). This yields the sugar hemiacetal product with overall retention of stereochemistry. The releasing of a dimeric sugar product (NAG at subsite −1, −2, as shown in Figure 4) may be indicated by elongation of the H-bond distance of HN2–Oδ_2:Asp142 from 2.13 Å to 2.21 Å (Table 2).

CONCLUSIONS

In this study, the deglycosylation step of chitin hydrolysis catalysed by *S. marcescens* ChiB was investigated by hybrid QM/MM calculation based on the available crystal structure of ChiB. The QM/MM PES calculated at AM1/CHARMM22 level was carried out using adiabatic mapping to track the possible reaction pathway and locate and characterise the structures corresponding to the stationary points. The PES showed the presence of two transition states, indicating that the deglycosylation path follows a stepwise mechanism in which the nucleophilic attack of water at the anomeric carbon occurs as the first step, followed by proton abstraction from water molecule to the

oxygen atom of Glu144. These results differ from the current belief that the deglycosylation should occur via a single transition state. The calculated barriers for the two transition states suggest that the nucleophilic attack of water molecule is the rate-limiting step for the deglycosylation. In addition, an oxocarbenium-like character in the first transition state was also captured. Such ionic character, as suggested by our QM/MM study, is likely to occur in both glycosylation and deglycosylation by ChiB, which seems to support previous findings that the formation of an oxocarbenium ion is a major characteristic of the hydrolysis of the β-glycosidic linkage.

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Full Paper

A novel method for estimating the parameter of a Gaussian AR(1) process with additive outliers

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Abstract: A novel estimator for a Gaussian first-order autoregressive [AR(1)] process with additive outliers is presented. A recursive median adjustment based on an α-trimmed mean was applied to the weighted symmetric estimator. The following estimators were considered: the weighted symmetric estimator ($\hat{\rho}_W$), the recursive-mean-adjusted weighted symmetric estimator ($\hat{\rho}_{R-W}$), the recursive-median-adjusted weighted symmetric estimator ($\hat{\rho}_{Rmd-W}$), and the weighted symmetric estimator using adjusted recursive median based on the α-trimmed mean ($\hat{\rho}_{Tm-Rmd-W}$). Using Monte Carlo simulations, the mean square errors (MSE) of the estimators were compared. Simulation results showed that the proposed estimator, $\hat{\rho}_{Tm-Rmd-W}$, provided a smaller MSE than those from $\hat{\rho}_W$, $\hat{\rho}_{R-W}$ and $\hat{\rho}_{Rmd-W}$ for almost all situations.

Keywords: parameter estimation, AR(1) process, recursive median, trimmed mean, additive outliers

INTRODUCTION

In time series analysis, outliers or aberrant observations can have adverse impact on model identification, parameter estimation as well as forecasting. Outliers may occur because of human error in such activity as typing, recording and measuring mistakes or because of abrupt, short-term changes in the underlying process [1]. Fox [2], Abraham and Box [3] and Martin [4] discussed two kinds of outliers that can be found in time series data, namely additive outliers (AO) and innovational outliers (IO). An additive outlier corresponds to the situation in which a gross error of observation or recording error affects a single observation [2]. An innovational outlier affects not only the particular observation, but also subsequent observations [2]. In this study, the additive outliers are focused on

because they are more harmful than innovational outliers [5]. A time series that does not contain any outliers is called an outlier-free series.

Suppose an outlier-free time series $\{X_t ; t = 2,3,...,n\}$ follows a Gaussian first-order autoregressive process, AR(1), satisfying

$$X_t = \mu + \rho(X_{t-1} - \mu) + e_t, \quad (1)$$

where μ is the mean of the process, ρ is an autoregressive parameter, and $|\rho| < 1$ and e_t are independent and identically distributed random variables having normal distribution with zero mean and variance σ_e^2, i.e. $e_t \sim N(0, \sigma_e^2)$. For $\rho = 1$, the model (1) is called the random walk model; otherwise it is called a stationary AR(1) process when $|\rho| < 1$. The model (1) will be called a random walk model if $|\rho| = 1$; otherwise it is called a stationary model. In the case of ρ being close to one or near a non-stationary model, the mean and variance of this model change over time. Let the observed time series be denoted by $\{Y_t\}$. An additive outlier model is defined as

$$Y_t = X_t + \delta I_t^{(T)}, \quad (2)$$

where δ is the magnitude of the additive outliers and $I_t^{(T)}$ is an indicator variable such that $I_t^{(T)} = 1$ if $t = T$, and $I_t^{(T)} = 0$ if $t \neq T$. The model (2) can be interpreted that $\{X_t\}$ has additive outliers at time point T $(1 < T < n)$.

One of the well-known estimators of ρ is the ordinary least square (OLS) estimator. Although the OLS estimator has asymptotic normality for $|\rho| < 1$ [6-7], it has long been known that the OLS estimator can have a large bias [8-10]. In addition, Conover [11] indicated that the OLS estimator is sensitive to outliers. Therefore, useful improvements in the parameter estimation have been proposed so as to reduce the bias of the OLS estimator. Park and Fuller [12] proposed the weighted symmetric estimator (W) of ρ. A robust estimator for an autoregressive model was presented by Denby and Martin [13]. Guo [14] developed a simple and robust estimator for an AR(1) model. So and Shin [15] applied a recursive mean adjustment to the OLS estimator (ROLS) and they found that the mean square error of the ROLS estimator is smaller than the OLS estimator for $\rho \in (0,1)$. They also showed that the ROLS estimator has a coverage probability which is close to the nominal value. Niwitpong [16] applied the recursive mean adjustment to the weighted symmetric estimator (R-W) of Park and Fuller [12]. Panichkitkosolkul [17] proposed an estimator for an unknown mean Gaussian AR(1) model with additive outliers by applying the recursive median adjustment to the weighted symmetric estimator (Rmd-W). He found that the Rmd-W estimator is more efficient than the W or R-W estimator in terms of the mean square error for almost all situations.

In this paper, a new recursive median adjustment based on an α-trimmed mean [18] is applied to the weighted symmetric estimator (abbreviated Tm-Rmd-W) for model (1) when there are additive outliers in time series data. Because the outliers do not affect the trimmed mean and median values, the recursive mean adjustment is replaced by the new recursive median adjustment based on the α-trimmed mean. The aim of this paper is to compare four estimators, i.e. the weighted symmetric estimator ($\hat{\rho}_W$), the weighted symmetric estimator based on the recursive mean adjustment ($\hat{\rho}_{R-W}$), the weighted symmetric estimator based on the recursive median adjustment ($\hat{\rho}_{Rmd-W}$), and the

weighted symmetric estimator based on the recursive median adjustment by using the trimmed mean ($\hat{\rho}_{Tm-Rmd-W}$), in terms of mean square error (MSE) of the estimators.

METHODOLOGY

Park and Fuller [12] proposed the weighted symmetric estimator of ρ given by

$$\hat{\rho}_W = \frac{\sum_{t=2}^{n}(Y_t-\bar{Y})(Y_{t-1}-\bar{Y})}{\sum_{t=3}^{n}(Y_{t-1}-\bar{Y})^2+n^{-1}\sum_{t=1}^{n}(Y_t-\bar{Y})^2}. \quad (3)$$

Niwitpong [16] replaces $\bar{Y}$ by $\bar{Y}_t=\frac{1}{t}\sum_{i=1}^{t}Y_i$ in (3). The estimator of ρ obtained as a result of this recursive mean adjustment is

$$\hat{\rho}_{R-W} = \frac{\sum_{t=2}^{n}(Y_t-\bar{Y}_t)(Y_{t-1}-\bar{Y}_{t-1})}{\sum_{t=3}^{n}(Y_{t-1}-\bar{Y}_{t-1})^2+n^{-1}\sum_{t=1}^{n}(Y_t-\bar{Y}_t)^2}. \quad (4)$$

When there are outliers in time series data, it affects the recursive mean $\bar{Y}_t$ in (4). Panichkitkosolkul [17] replaces the recursive mean in (4) by the recursive median, $\tilde{Y}_t$. The estimator of ρ obtained as a result of the recursive median adjustment is

$$\hat{\rho}_{Rmd-W} = \frac{\sum_{t=2}^{n}(Y_t-\tilde{Y}_t)(Y_{t-1}-\tilde{Y}_{t-1})}{\sum_{t=3}^{n}(Y_{t-1}-\tilde{Y}_{t-1})^2+n^{-1}\sum_{t=1}^{n}(Y_t-\tilde{Y}_t)^2}, \quad (5)$$

where $\tilde{Y}_t = median(Y_1, Y_2, ..., Y_t)$.

The effect of additive outliers on an estimator of ρ in model (1) can be reduced by using new recursive median adjustment based on an α-trimmed mean. The proposed recursive median values adjusted by an α-trimmed mean are derived from computing the α-trimmed mean of the recursive median. Therefore, the recursive median in (5) is replaced by a new recursive median. A novel estimator of ρ obtained as a result of this new recursive median adjustment is given by

$$\hat{\rho}_{Tm-Rmd-W} = \frac{\sum_{t=2}^{n}(Y_t-\bar{\tilde{Y}}_t)(Y_{t-1}-\bar{\tilde{Y}}_{t-1})}{\sum_{t=3}^{n}(Y_{t-1}-\bar{\tilde{Y}}_{t-1})^2+n^{-1}\sum_{t=1}^{n}(Y_t-\bar{\tilde{Y}}_t)^2}, \quad (6)$$

where $\bar{\tilde{Y}}_t=\frac{1}{t-2[t\alpha]}\sum_{i=[t\alpha]+1}^{t-[t\alpha]}\tilde{Y}_{(i)}$; $\tilde{Y}_{(i)}$ denotes the ordered values of the recursive median $\tilde{Y}_i$, i.e. $\tilde{Y}_{(1)} \le \tilde{Y}_{(2)} \le \cdots \le \tilde{Y}_{(t)}$; α denotes the proportion of observations removed from both the upper and lower bounds, $0 < \alpha < 0.5$; and $[t\alpha]$ denotes the greatest integer not greater than $t\alpha$.

The performance of the proposed estimator for a Gaussian AR(1) process with additive outliers was examined via Monte Carlo simulations with particular emphasis on comparison between the novel and existing approaches. Data were generated from a Gaussian AR(1) process with additive outliers.

[$Y_1 \sim N(0, \frac{\sigma_e^2}{1-\rho^2})$ was generated and the time series of length $n+50$ was simulated but the time series used in calculations were $\{Y_{51}, Y_{52}, ..., Y_{n+50}\}$.] The following parameter values were used: (μ, σ_e)= (0, 1); ρ = 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 0.9; sample sizes, n= 25, 50, 100 and 250; magnitude of AO effect, $\delta = 3\sigma_e$ and $5\sigma_e$; percentage of additive outliers, $p = 5\%$ and 10%; and fraction of data to be trimmed, $\alpha = 0.05$. All simulations were performed using programs written in R statistical software [19-21] with the number of simulation runs, $M = 10,000$. In addition, the additive outliers occurred randomly. The Monte Carlo simulation results of estimating MSE of these estimators, $\hat{\rho}_W$, $\hat{\rho}_{R-W}$, $\hat{\rho}_{Rmd-W}$ and $\hat{\rho}_{Tm-Rmd-W}$, are presented in the next section.

RESULTS

The simulation results are shown in Tables 1-2. The estimated MSE of all estimators, $\hat{\rho}_W$, $\hat{\rho}_{R-W}$, $\hat{\rho}_{Rmd-W}$ and $\hat{\rho}_{Tm-Rmd-W}$, is given by

$$MSE = Variance + Bias^2 = \frac{\sum_{j=1}^{M}(\hat{\rho}_j - \bar{\hat{\rho}})^2}{M-1} + \left[E(\hat{\rho}) - \rho\right]^2, \quad (7)$$

where $E(\hat{\rho}) = \bar{\hat{\rho}} = M^{-1}\sum_{j=1}^{M}\hat{\rho}_j$. As can be seen from Tables 1-2, the MSE of $\hat{\rho}_W$ is larger than those of the other estimators, especially when ρ is close to one and for small sample sizes. These values of MSE decrease as sample size gets larger (0.0412−0.1163 for $n = 25$; 0.0217−0.0507 for $n = 50$; 0.0114−0.0359 for $n = 100$; and 0.0050−0.0228 for $n = 250$). The $\hat{\rho}_W$ performs well for $n \geq 50$. On the other hand, the novel estimator, $\hat{\rho}_{Tm-Rmd-W}$, provides the lowest MSE in all scenarios that were considered except when the parameter ρ is small ($\rho = 0.1$ or 0.2). Additionally, $\hat{\rho}_{Tm-Rmd-W}$ performs very well in relation to the other three estimators. The proposed estimator, $\hat{\rho}_{Tm-Rmd-W}$ in (6), dominates all estimators since its MSE is the lowest for almost all cases. One reason for this is that the additive outliers do not affect the median and α-trimmed mean values. Moreover, the adjusted recursive median values applied in the formula of $\hat{\rho}_{Tm-Rmd-W}$ in (6) can also reduce the MSE of the estimator. For other estimators, the MSE of $\hat{\rho}_{Rmd-W}$ is less than that of $\hat{\rho}_{R-W}$ and $\hat{\rho}_W$ for almost all situations. The $\hat{\rho}_{Rmd-W}$ often ranks the second best after the proposed estimator. Furthermore, the values of MSE shown in Table 1 are less than those reported in Table 2 because the time series data in Table 1 have fewer outliers.

Using Monte Carlo simulations, the densities of estimates of the estimators, $\hat{\rho}_W$, $\hat{\rho}_{R-W}$, $\hat{\rho}_{Rmd-W}$ and $\hat{\rho}_{Tm-Rmd-W}$, are plotted in Figures 1-3 for each of $\rho = 0.2, 0.5, 0.9$, $n = 100$, $p = 10\%$ and $\delta = 3\sigma_e$. As can be seen from these figures, the estimated densities of all estimators are not different and they are symmetric when $\rho = 0.2$ and 0.5. However, when ρ is equal to 0.9, the density estimates of all estimators are skewed to the left. In addition, the difference between the mode of the estimated density of $\hat{\rho}_{Tm-Rmd-W}$ and true parameter ρ is smallest compared to those obtained with other estimators.

Table 1. Estimated values of MSE of $\hat{\rho}_W$, $\hat{\rho}_{R-W}$, $\hat{\rho}_{Rmd-W}$ and $\hat{\rho}_{Tm-Rmd-W}$ when percentage of additive outliers, $p = 5\%$

n	ρ	$\delta = 3\sigma_e$				$\delta = 5\sigma_e$			
		W	R-W	Rmd-W	Tm-Rmd-W	W	R-W	Rmd-W	Tm-Rmd-W
25	0.1	0.0412	0.0367	0.0346	0.0428	0.0394	0.0347	0.0318	0.0339
	0.2	0.0487	0.0417	0.0389	0.0407	0.0539	0.0456	0.0420	0.0357
	0.3	0.0597	0.0500	0.0471	0.0412	0.0767	0.0647	0.0609	0.0461
	0.4	0.0714	0.0594	0.0562	0.0418	0.1040	0.0887	0.0849	0.0600
	0.5	0.0825	0.0693	0.0664	0.0453	0.1309	0.1129	0.1102	0.0756
	0.6	0.0956	0.0810	0.0791	0.0509	0.1640	0.1433	0.1422	0.0964
	0.7	0.1053	0.0900	0.0893	0.0550	0.1933	0.1705	0.1690	0.1125
	0.8	0.1106	0.0963	0.0963	0.0581	0.2093	0.1861	0.1851	0.1216
	0.9	0.1163	0.1038	0.1038	0.0626	0.2221	0.1994	0.2002	0.1308
50	0.1	0.0217	0.0200	0.0191	0.0236	0.0220	0.0202	0.0189	0.0197
	0.2	0.0252	0.0226	0.0209	0.0214	0.0335	0.0300	0.0276	0.0229
	0.3	0.0310	0.0276	0.0259	0.0223	0.0481	0.0431	0.0400	0.0296
	0.4	0.0377	0.0334	0.0312	0.0232	0.0672	0.0608	0.0575	0.0399
	0.5	0.0443	0.0395	0.0377	0.0255	0.0881	0.0804	0.0777	0.0532
	0.6	0.0491	0.0442	0.0430	0.0270	0.1044	0.0963	0.0936	0.0628
	0.7	0.0507	0.0460	0.0450	0.0270	0.1176	0.1087	0.1068	0.0701
	0.8	0.0493	0.0453	0.0447	0.0255	0.1177	0.1100	0.1084	0.0691
	0.9	0.0440	0.0415	0.0412	0.0233	0.1058	0.0998	0.0988	0.0610
100	0.1	0.0114	0.0107	0.0101	0.0116	0.0139	0.0130	0.0114	0.0111
	0.2	0.0152	0.0140	0.0128	0.0116	0.0247	0.0230	0.0197	0.0157
	0.3	0.0206	0.0190	0.0175	0.0133	0.0403	0.0378	0.0337	0.0252
	0.4	0.0262	0.0243	0.0226	0.0155	0.0585	0.0553	0.0511	0.0380
	0.5	0.0315	0.0294	0.0277	0.0180	0.0788	0.0750	0.0707	0.0522
	0.6	0.0359	0.0337	0.0323	0.0204	0.0948	0.0908	0.0867	0.0632
	0.7	0.0358	0.0338	0.0325	0.0197	0.1039	0.0998	0.0964	0.0680
	0.8	0.0318	0.0303	0.0293	0.0172	0.0977	0.0940	0.0915	0.0620
	0.9	0.0229	0.0222	0.0217	0.0121	0.0719	0.0695	0.0677	0.0427
250	0.1	0.0050	0.0048	0.0045	0.0046	0.0072	0.0069	0.0055	0.0050
	0.2	0.0080	0.0076	0.0068	0.0054	0.0160	0.0154	0.0127	0.0102
	0.3	0.0119	0.0113	0.0103	0.0074	0.0298	0.0289	0.0253	0.0203
	0.4	0.0164	0.0157	0.0146	0.0102	0.0462	0.0450	0.0410	0.0333
	0.5	0.0201	0.0194	0.0183	0.0126	0.0624	0.0610	0.0570	0.0462
	0.6	0.0228	0.0221	0.0212	0.0144	0.0752	0.0737	0.0701	0.0561
	0.7	0.0224	0.0218	0.0210	0.0138	0.0792	0.0777	0.0750	0.0585
	0.8	0.0176	0.0172	0.0167	0.0105	0.0685	0.0673	0.0654	0.0481
	0.9	0.0101	0.0100	0.0097	0.0056	0.0409	0.0403	0.0391	0.0261

Table 2. Estimated values of MSE of $\hat{\rho}_W$, $\hat{\rho}_{R-W}$, $\hat{\rho}_{Rmd-W}$ and $\hat{\rho}_{Tm-Rmd-W}$ when percentage of additive outliers, $p = 10\%$

n	ρ	$\delta = 3\sigma_e$				$\delta = 5\sigma_e$			
		W	R-W	Rmd-W	Tm-Rmd-W	W	R-W	Rmd-W	Tm-Rmd-W
25	0.1	0.0443	0.0395	0.0363	0.0413	0.0439	0.0388	0.0335	0.0343
	0.2	0.0570	0.0484	0.0440	0.0411	0.0663	0.0571	0.0493	0.0421
	0.3	0.0735	0.0616	0.0560	0.0443	0.0995	0.0862	0.0765	0.0612
	0.4	0.0927	0.0783	0.0730	0.0532	0.1410	0.1237	0.1125	0.0859
	0.5	0.1159	0.0991	0.0936	0.0639	0.1869	0.1654	0.1546	0.1165
	0.6	0.1379	0.1193	0.1141	0.0759	0.2291	0.2045	0.1924	0.1430
	0.7	0.1529	0.1334	0.1298	0.0841	0.2821	0.2542	0.2437	0.1781
	0.8	0.1700	0.1496	0.1467	0.0941	0.3176	0.2877	0.2776	0.2010
	0.9	0.1754	0.1568	0.1550	0.0987	0.3370	0.3059	0.2971	0.2101
50	0.1	0.0241	0.0222	0.0206	0.0229	0.0272	0.0250	0.0202	0.0208
	0.2	0.0337	0.0301	0.0266	0.0234	0.0450	0.0409	0.0317	0.0274
	0.3	0.0469	0.0419	0.0373	0.0281	0.0749	0.0687	0.0557	0.0450
	0.4	0.0622	0.0560	0.0504	0.0355	0.1093	0.1013	0.0860	0.0677
	0.5	0.0804	0.0731	0.0677	0.0461	0.1515	0.1417	0.1259	0.0987
	0.6	0.0936	0.0858	0.0808	0.0536	0.1888	0.1773	0.1611	0.1238
	0.7	0.1032	0.0954	0.0910	0.0594	0.2228	0.2102	0.1955	0.1465
	0.8	0.1035	0.0962	0.0929	0.0591	0.2365	0.2233	0.2119	0.1529
	0.9	0.0912	0.0859	0.0836	0.0511	0.2228	0.2110	0.2022	0.1385
100	0.1	0.0132	0.0123	0.0109	0.0118	0.0161	0.0151	0.0110	0.0114
	0.2	0.0206	0.0190	0.0162	0.0134	0.0324	0.0304	0.0218	0.0185
	0.3	0.0312	0.0290	0.0253	0.0185	0.0581	0.0551	0.0425	0.0350
	0.4	0.0434	0.0407	0.0364	0.0256	0.0890	0.0852	0.0702	0.0574
	0.5	0.0563	0.0531	0.0488	0.0337	0.1243	0.1197	0.1041	0.0849
	0.6	0.0674	0.0640	0.0602	0.0411	0.1576	0.1522	0.1371	0.1103
	0.7	0.0700	0.0668	0.0637	0.0425	0.1804	0.1746	0.1620	0.1270
	0.8	0.0652	0.0625	0.0602	0.0383	0.1821	0.1762	0.1664	0.1239
	0.9	0.0469	0.0454	0.0439	0.0262	0.1437	0.1393	0.1333	0.0912
250	0.1	0.0064	0.0061	0.0049	0.0048	0.0093	0.0090	0.0052	0.0052
	0.2	0.0126	0.0121	0.0097	0.0076	0.0244	0.0236	0.0152	0.0133
	0.3	0.0222	0.0214	0.0183	0.0139	0.0473	0.0462	0.0339	0.0295
	0.4	0.0328	0.0318	0.0283	0.0215	0.0772	0.0757	0.0610	0.0538
	0.5	0.0443	0.0431	0.0398	0.0306	0.1088	0.1070	0.0914	0.0802
	0.6	0.0518	0.0505	0.0477	0.0361	0.1381	0.1361	0.1213	0.1054
	0.7	0.0533	0.0521	0.0500	0.0369	0.1568	0.1546	0.1424	0.1208
	0.8	0.0452	0.0443	0.0427	0.0300	0.1500	0.1478	0.1402	0.1131
	0.9	0.0254	0.0251	0.0243	0.0154	0.1002	0.0987	0.0951	0.0695

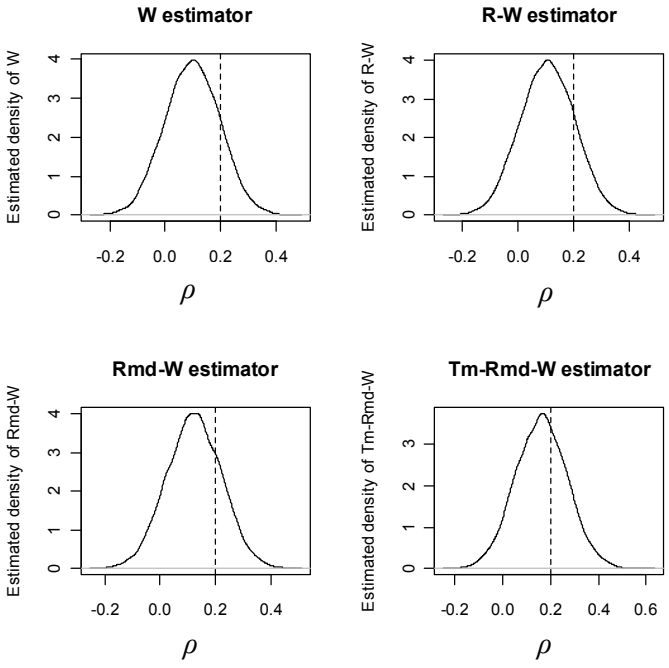

Figure 1. Comparison of all density estimates when $\rho = 0.2$, $n = 100$, $p = 10\%$ and $\delta = 3\sigma_e$

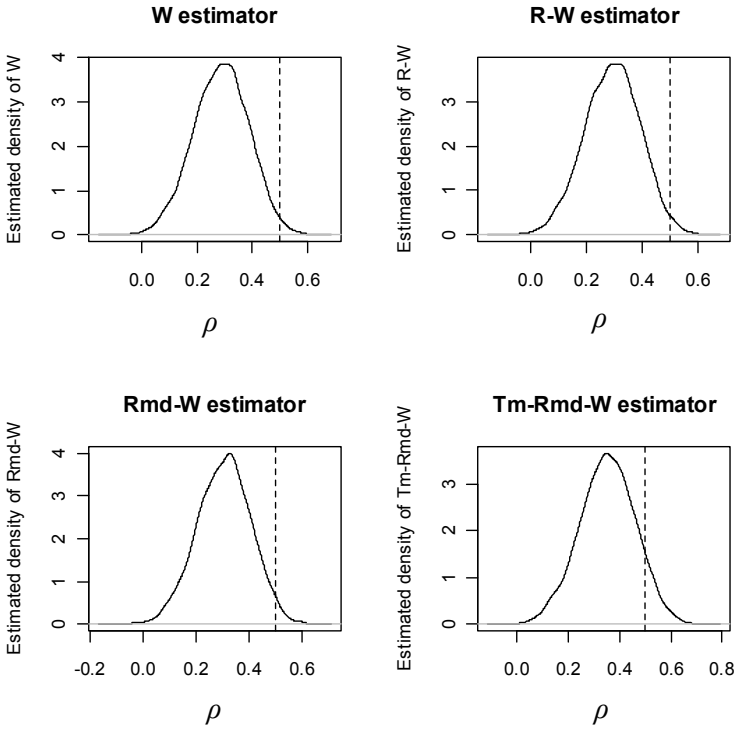

Figure 2. Comparison of all density estimates when $\rho = 0.5$, $n = 100$, $p = 10\%$ and $\delta = 3\sigma_e$

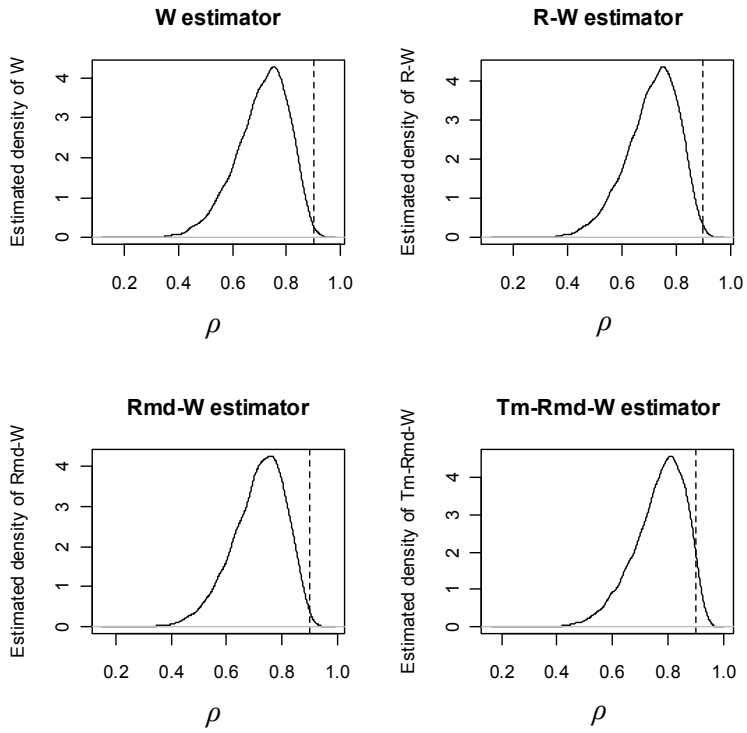

Figure 3. Comparison of all density estimates when $\rho = 0.9$, $n = 100$, $p = 10\%$ and $\delta = 3\sigma_e$

REAL DATA EXAMPLE

To illustrate the application of the estimators which have been proposed in the previous section, the yearly real exchange rates between USA and Sudan from 1970 to 2008 (base year: 2005) are used. A series giving a total of 39 observations was collected from the Economic Research Service, United States Department of Agriculture [22]. The time series plot, the sample autocorrelation function (ACF) and the sample partial autocorrelation function (PACF), as shown in Figures 4-5, suggest that an AR(1) model is suitable. The additive outliers of this series were detected by using an iterative detecting procedure proposed by Chang et al. [23] via the R statistical software [1]. It was found that the time indices of potential AO are $t = 22$ and 23 (year 1991 and 1992). All estimators, $\hat{\rho}_W$, $\hat{\rho}_{R-W}$, $\hat{\rho}_{Rmd-W}$ and $\hat{\rho}_{Tm-Rmd-W}$ and their standard errors and variances were also constructed (Table3). As presented in Table 4, the proposed estimator, $\hat{\rho}_{Tm-Rmd-W}$, provides about 11.6%, 11.1% and 10.0% less standard error than those of the $\hat{\rho}_W$, $\hat{\rho}_{R-W}$ and $\hat{\rho}_{Rmd-W}$ respectively, which confirms that the proposed estimator, $\hat{\rho}_{Tm-Rmd-W}$, is much better than the other estimators.

Figure 4. The US/Sudan annual real exchange rates from 1970 to 2008

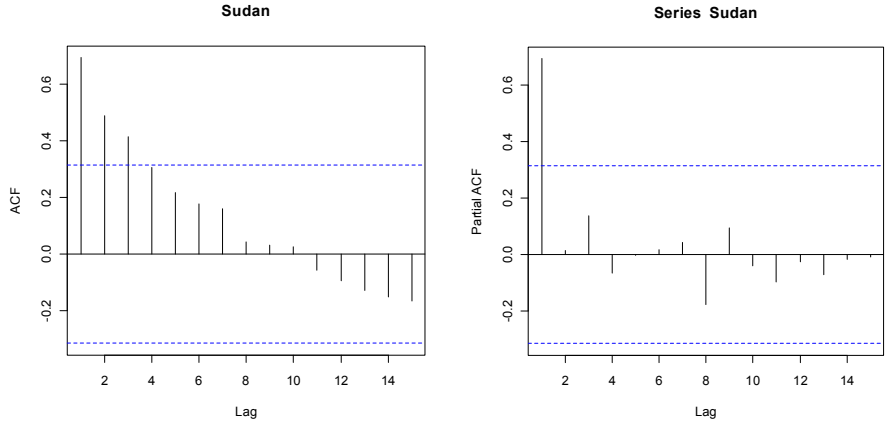

Figure 5. ACF and PACF of the US/Sudan real exchange rates

Table 3. The standard errors and variances of all estimators

$SE(\hat{\rho}_W) = \dfrac{\hat{\sigma}_W}{\sqrt{\sum_{t=2}^{n}(Y_{t-1}-\bar{Y})^2}}$,	$\hat{\sigma}_W^2 = \dfrac{\sum_{t=2}^{n}(Y_t-\bar{Y}-\hat{\rho}_W(Y_{t-1}-\bar{Y}))^2}{n-2}$
$SE(\hat{\rho}_{R-W}) = \dfrac{\hat{\sigma}_{R-W}}{\sqrt{\sum_{t=2}^{n}(Y_{t-1}-\bar{Y}_{t-1})^2}}$,	$\hat{\sigma}_{R-W}^2 = \dfrac{\sum_{t=2}^{n}(Y_t-\bar{Y}_t-\hat{\rho}_{R-W}(Y_{t-1}-\bar{Y}_{t-1}))^2}{n-2}$
$SE(\hat{\rho}_{Rmd-W}) = \dfrac{\hat{\sigma}_{Rmd-W}}{\sqrt{\sum_{t=2}^{n}(Y_{t-1}-\tilde{Y}_{t-1})^2}}$,	$\hat{\sigma}_{Rmd-W}^2 = \dfrac{\sum_{t=2}^{n}(Y_t-\tilde{Y}_t-\hat{\rho}_{Rmd-W}(Y_{t-1}-\tilde{Y}_{t-1}))^2}{n-2}$
$SE(\hat{\rho}_{Tm-Rmd-W}) = \dfrac{\hat{\sigma}_{Tm-Rmd-W}}{\sqrt{\sum_{t=2}^{n}(Y_{t-1}-\bar{\tilde{Y}}_{t-1})^2}}$,	$\hat{\sigma}_{Tm-Rmd-W}^2 = \dfrac{\sum_{t=2}^{n}(Y_t-\bar{\tilde{Y}}_t-\hat{\rho}_{Tm-Rmd-W}(Y_{t-1}-\bar{\tilde{Y}}_{t-1}))^2}{n-2}$

Table 4. Parameter estimates and standard errors of estimators for US/Sudan real exchange rates series

Method	Estimate	Standard error (SE)
W	0.6766	0.11871
R-W	0.6799	0.11820
Rmd-W	0.6860	0.11706
Tm-Rmd-W	0.7435	0.10641

CONCLUSIONS

A novel estimator for a Gaussian AR(1) process with additive outliers has been proposed. This estimator of the autoregressive parameter is obtained by applying a recursive median adjustment based on an α-trimmed mean to the weighted symmetric estimator. The adjusted recursive median values are derived from computation of the α-trimmed mean of the recursive median. The weighted symmetric estimator ($\hat{\rho}_W$), the recursive-mean-adjusted weighted symmetric estimator ($\hat{\rho}_{R-W}$), the recursive-median-adjusted weighted symmetric estimator ($\hat{\rho}_{Rmd-W}$) and the novel estimator ($\hat{\rho}_{Tm-Rmd-W}$) are compared in this study. The result shows that the novel estimator gives the best performance in terms of the mean square error of the estimators for almost all scenarios.

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Technical Report

Conceptual design of motorcycle's lumbar support using motorcyclists' anthropometric characteristics

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Abstract: This study presents the design and development of a prototype of lumbar support for motorcyclists corresponding to their anthropometric dimensions. The total design process model was used for this purpose. The critical design dimensions for the lumbar support (height, width, adjustable range and thickness) were obtained from the anthropometric dimensions of motorcyclists (1032 samples). The initial testing (trial runs) of the prototype proved to be successful as it was capable of providing comfort to the motorcyclists' lumbar region during their riding process. However, further evaluation needs to be done in order to evaluate the stability, solidity, durability and safety of the prototype.

Keywords: design, ergonomics, anthropometrics, motorcycle, lumbar support

INTRODUCTION

'Engineering design' and 'ergonomics' are two important terms in the design process. The primary purpose of engineering design is to devise a system, component or process to meet the desired needs by utilising the basic knowledge (basic sciences, mathematics and engineering sciences)

and the available resources. The International Ergonomics Association (IEA) defines ergonomics as the discipline that involves the understanding of the interaction between humans and other elements of a system and the profession that applies theory, principles, data and methods to designing in order to optimise human well-being and overall system performance [1]. Thus, the primary purpose of the interdisciplinary subject of engineering design and ergonomic design is to devise a system or product with added human value.

There are four basic criteria in an ergonomic product design, namely increasing production, decreasing injuries, decreasing human error and increasing user satisfaction [2-3]. These criteria are applied to the relevant industries to meet particular human needs. However, to design a product or system that can accomplish these criteria is very demanding as the advancement in technology has made the products/systems more sophisticated and complex. An example of such a complex (human-machine) interaction can be seen in the production of motor vehicles, especially motorcycles [4].

The ergonomic design involving motorcycles is a complex process as it involves a very constrained space between the motorcyclist and the motorcycle. In any adjustment of the design of the motorcycle, the different needs of the motorcyclist must be considered [5-7]. Generally, the main aspect of a motorcycle design is to provide for the safety and comfort of the motorcyclist by reducing or eliminating fatigue during the riding process. Previous research [8] has shown that motorcyclists in Malaysia experience during the riding process symptoms of discomfort on various parts of their bodies, particularly the lower part of the back (lumbar) area. Similarly, other researchers have also found that sitting for a prolonged duration of time in a vehicle can cause great intradiscal pressure in the lumbar region and consequent low back pain [2, 9-10]. The lumbar region is also the most vulnerable part of the spine as this part is suspended between the upper heavy part of the body including the rib cage and the lower and lighter part starting from the hip bone [11].

Therefore, this lumbar region should be supported by a backrest. However, in Malaysia the current design of motorcycles does not incorporate this feature. Consequently, motorcyclists assume a variety of postures (Figure 1) during their riding to balance the intradiscal pressure in their lumbar region. In our earlier study, we managed to design and develop a prototype of portable back support [12]. However, the developed design was lacking in some important ergonomic characteristics, i.e. anthropometric dimensions, owing to the unavailability of this information during the study period. This study is undertaken to design and develop an improved version of the earlier lumbar support prototype for motorcyclists by taking into consideration their anthropometric dimensions.

METHODS

The design and development of a new lumbar support for motorcyclists is based on Pugh's total design process model [13]. There are six important components in this model, viz. market study, product design specification (PDS), conceptual design, detail design, manufacturing, and sales (Figure 2). An important guide in designing the lumbar support is the information on anthropometric dimensions of the motorcyclists, which are needed to ensure that the designed product can be adapted to suit the majority of users (5^{th} percentile to the 99^{th} percentile).

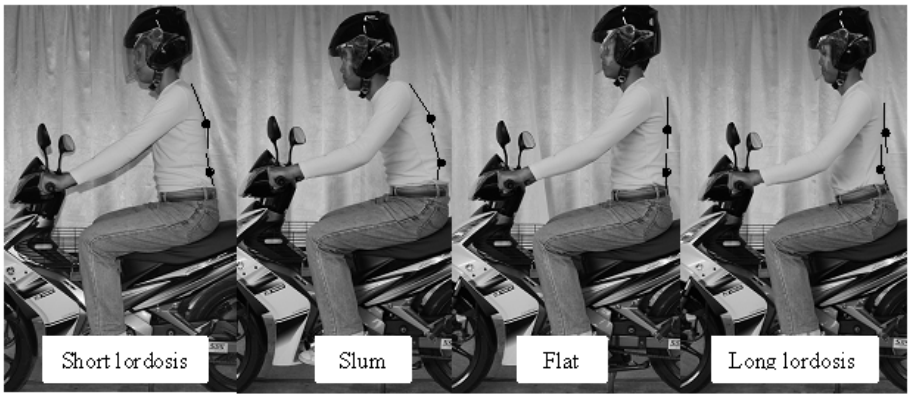


Figure 1. Variety of riding postures

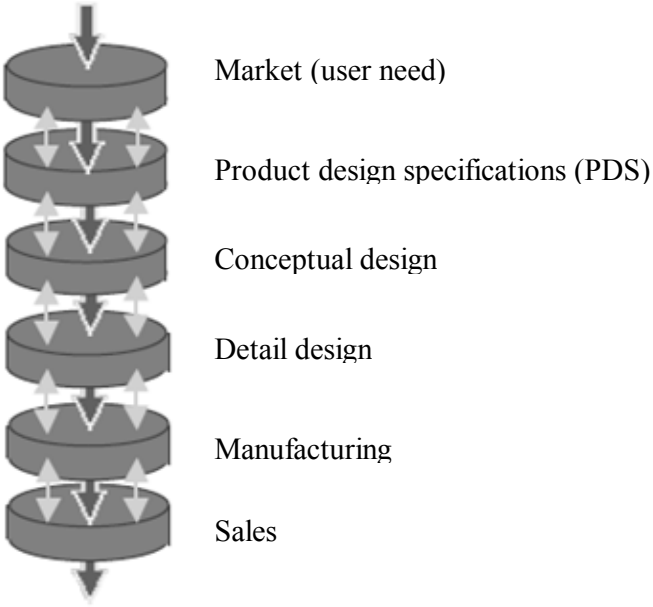


Figure 2. Total design process model

The anthropometric data were obtained from an earlier survey conducted in the Polytechnic of Sultan Azlan Shah in Malaysia [14]. The data were collected and analysed based on Malaysian standards [15-16]. The sample consisted of 1032 students (595 males and 437 females). Their ages ranged from 18 to 24 years, with a mean of 19.82 years and a standard deviation of 1.07. A total of 11 anthropometric dimensions were extracted in line with the current study's purpose (Table 1 and Figure 3).

Table 1. List of body dimensions selected for measurement including age and weight

Dimension Number	Dimension	Description
1	Age (year)	
2	Weight (kg)	Total mass (weight) of the body
3	Stature	Vertical distance from the floor to the highest point of the head (vertex).
4	Shoulder (biacromial) breadth	Distance along a straight line from acromion to acromion
5	Hip Breadth, sitting	Breadth of the body measured across the widest portion of the hips
6	Shoulder height, sitting	Vertical distance from a horizontal sitting surface to the acromion
7	Elbow height, sitting	Vertical distance from a horizontal sitting surface to the lowest bony point of the elbow when it is bent at a right angle with the forearm horizontal
8	Buttock-popliteal length (seat depth)	Horizontal distance from the hollow of the knee to the rearmost point of the buttock
9	Lower leg length (popliteal height)	Vertical distance from the footrest surface to the lower surface of the thigh immediately behind the knee, bent at right angles
10	Upper hip bone height, sitting	Distance from floor to the uppermost point of the left hipbone. The hipbone is traced by palpating [11, 16].
11	Lowest rib bone height, sitting	Distance from floor to the bottom of the lowest left rib. The lowest left rib is traced by palpating [11, 16].

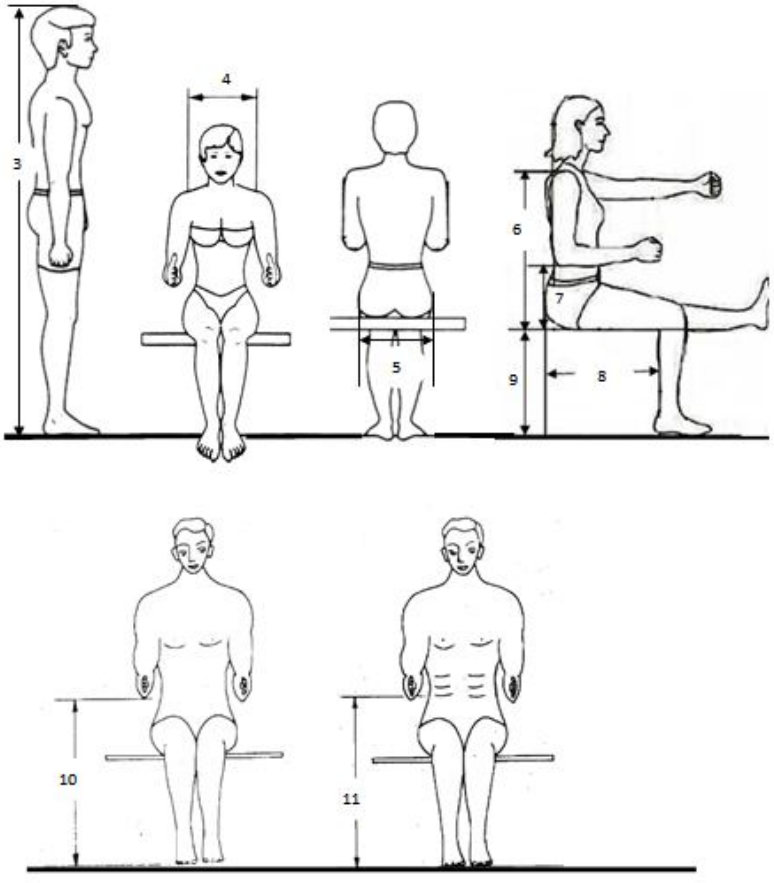


Figure 3. Illustrations of anthropometric dimensions corresponding to Table 1

Product design specifications (PDS) are used for analysis, design, manufacturing and construction of a structure or a component in order to achieve a specified degree of safety, efficiency, performance or quality as well as a common standard of good design practice [17]. A total of six PDS criteria, viz. safety, material, weight, performance, installation and ergonomics, were chosen for the development of the lumbar support (Table 2).

Table 2. Product design specifications for lumbar support

No.	Criterion	Specification
1	Performance	• Can support maximum body weight of 120 kg • Can be adjustable upward and downward according to lumbar height • Can be adjustable forward and backward according to rider's comfort • A good rigid frame
2	Safety	• Should obey the legislation of the local road safety requirements • Should not harm the rider or other road users
3	Installation	• Should fit to seat dimensions of present motorbike • Can be easily fixed with the existing holes and lugs in the motorcycle • Installation can be done using simple tools (such as screwdriver or spanner)
4	Weight	• Below 5 kg
5.	Material	• Light • Strong • Anti-Rust • Easy to form shape • Low cost • Easy to machine
6.	Ergonomics	• Cushion (contour shape) will support the back posture • Design features dimension based on the anthropometric dimensions of the motorcyclists • No sharp edges

In the conceptual design stage, conceptual sketches based on the PDS requirements are generated. A total of three conceptual designs (Figures 4-6) were developed for the lumbar support with detailed characteristics as described in Table 3. The matrix method [13] was used to select the best conceptual design. This method compares the generated conceptual designs, one with the other, against the criteria of evaluation (PDS). The result is shown in Table 4. The best conceptual design (with the highest score of +'s) was selected and then forwarded to the detail design section. In this case, Design 3 with the best performance, safety, weight, material and ergonomics was selected.

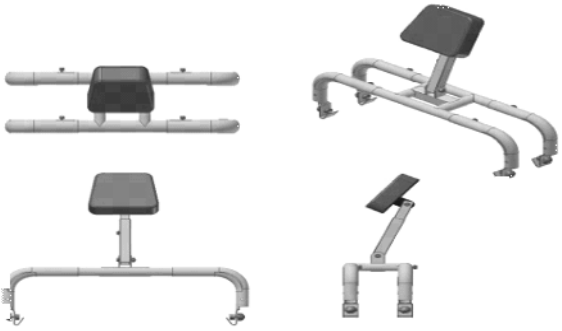


Figure 4. Conceptual design 1

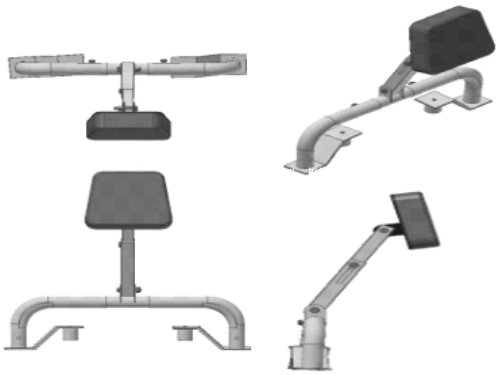


Figure 5. Conceptual design 2

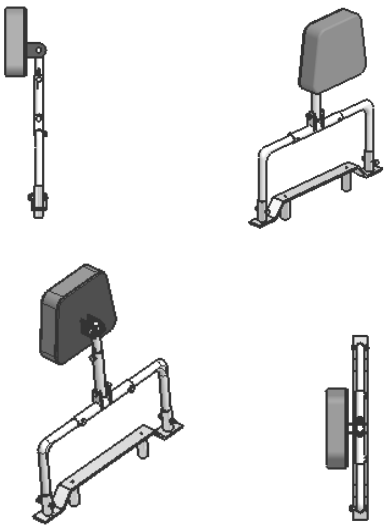
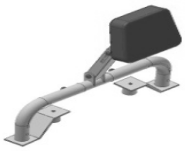
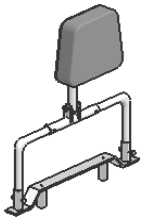


Figure 6. Conceptual design 3

Table 3. Characteristics of the conceptual designs

Characteristic	Design 1	Design 2	Design 3
Performance	• Can support the back posture (lumbar) during the riding process. • The height of support (upward and downward) can be adjusted. • The support can be adjusted forward and backward to suit the rider's comfort. • The angle of support (seat) can be adjusted. • Good rigid frame	• Can support the back posture (lumbar) during the riding process. • The height of support (upward and downward) can be adjusted. • The support can be adjusted forward and backward to suit the rider's comfort. • The angle of support (seat) can be adjusted. • Good rigid frame	• Can support the back posture (lumbar) during the riding process. • The height of support (upward and downward) can be adjusted. • The angle of support (seat) can be adjusted. • Good rigid body and base frame • The top frame (which consists of the support) can be removed from the bottom frame (which is fixed to the motorcycle) if desired.
Safety	• Does not offend the local road safety requirements.	• Does not offend the local road safety requirements. • The base frame is firmly fixed to the bottom of the motorcycle.	• Does not offend the local road safety requirements. • The base frame is firmly fixed to the bottom of the motorcycle.
Installation	• Can be easily fixed to current motorcycle's seat dimension. A separate belt and hook with slot concept is used to hold the base frame with the seat. • Simple tools are used to fix.	• Needs to be fixed to the motorcycle seat (at the underneath of the seat itself). • Simple tools are used to fix.	• Needs to be fixed to motorcycle seat. The bottom frame is fixed to the motorcycle body at the underneath of the seat while the top frame is slotted in from the top. • Simple tools are used to fix.
Weight	• In range of 5-6 kg	• In range of 4-5 kg	• In range of 4-5 kg
Material	• Alloy steel (frame) and foam (support)	• Aluminum (frame) and memory foam (support)	• Aluminum (frame) and memory foam (support)
Ergonomics	• Design based on anthropometric dimensions [14]	• Design based on anthropometric dimensions [14]	• Design based on anthropometric dimensions [14] • Can withstand greater force (user weight) due to body leaning on support.

Table 4. Conceptual design evaluation using the matrix method

Conceptual design / Criterion	1 	2 	3 
Performance	-	-	+
Safety	-	+	+
Installation	+	-	-
Weight	-	+	+
Material	-	+	+
Ergonomics	-	+	+
∑ +	1	4	5
∑ −	5	2	1

Note: + (plus) = better than; - (minus) = worse than; ∑ + = score of +'s; ∑ − = score of –'s

In the detail design, the results obtained from the anthropometric dimensions [mean, standard deviation (SD), standard error of the mean (SEM), coefficient of variation (CV), minimum value (Min), 1st percentile (1st), 5th percentile (5th), 50th percentile, 95th percentile, 99th percentile (99th) and maximum value (Max)] of motorcyclists, shown in Tables 5-7, were utilised. Based on these dimensions, important design features were determined (Table 8). The lumbar support should be 16.0 cm in height and 38.5 cm in width. It should also be adjustable between 14.2 -30.2 cm from the motorcycle seating surface. In addition, 5.0 cm was recommended as the minimum thickness for the lumbar support [9, 11]. The results of the detail design are shown in Figure 7.

Table 5. Anthropometric data for Malaysian males, aged 18–24 years (n= 595) [14]

No.	Measurement (cm)	Mean	SD	SEM	CV (%)	Min	1st	5th	50th	95th	99th	Max
1	Age (year)	19,70	1,00	0,04	5,05	18,00	18,00	19,00	19,00	21,00	23,00	24,00
2	Stature	168,01	6,08	0,25	3,62	150,50	152,90	159,38	167,40	178,34	183,50	186,18
3	Weight (kg)	64,33	15,2	0,62	23,6	41,00	43,00	46,00	60,00	99,00	115,08	120,00
4	Shoulder (biacromial) breadth	43,28	2,95	0,12	6,81	35,20	36,40	39,26	42,80	49,30	51,51	52,80
5	Hip Breadth, sitting	31,35	3,31	0,14	10,5	22,10	22,50	27,28	30,90	37,62	40,61	40,80
6	Shoulder height, sitting	55,74	3,21	0,13	5,77	46,70	47,28	50,50	55,60	61,42	63,81	65,30
7	Elbow height, sitting	19,20	3,25	0,13	16,9	11,80	12,70	14,50	18,90	25,40	27,42	40,50
8	Buttock-popliteal length (seat depth)	49,05	3,52	0,14	7,17	38,60	40,07	42,40	49,30	54,40	56,30	59,40
9	Lower leg length (popliteal height)	41,44	1,42	0,06	3,44	37,60	38,20	39,30	41,30	44,00	45,30	45,70
10	Upper hip bone height, sitting	56,43	3,60	0,15	6,38	47,60	49,19	51,00	56,20	62,52	65,90	76,30
11	Lowest rib bone height, sitting	67,55	4,11	0,17	6,09	58,20	59,79	61,48	67,20	74,82	78,51	89,60

Table 6. Anthropometric data for Malaysian females, aged 18–24 years (n= 437) [14]

No.	Measurement (cm)	Mean	SD	SEM	CV (%)	Min	1st	5th	50th	95th	99th	Max
1	Age (year)	19,98	1,14	0,05	5,71	18,00	19,00	19,00	19,00	22,00	24,00	24,00
2	Stature	156,07	5,32	0,25	3,41	141,50	143,08	146,49	155,90	163,91	170,06	170,70
3	Weight (kg)	55,88	10,7	0,51	19,1	36,00	38,00	41,00	55,00	76,00	92,24	100,00
4	Shoulder (biacromial) breadth	37,51	2,74	0,13	7,32	30,20	30,34	33,29	37,30	42,40	44,42	45,10
5	Hip Breadth, sitting	31,75	3,68	0,18	11,6	22,70	23,21	26,49	31,30	39,00	41,90	42,80
6	Shoulder height, sitting	52,32	4,17	0,20	7,97	42,40	42,83	44,49	52,30	60,01	63,30	64,70
7	Elbow height, sitting	19,30	3,21	0,15	16,6	11,40	12,15	14,30	18,90	24,91	26,96	27,80
8	Buttock-popliteal length (seat depth)	45,70	3,82	0,18	8,35	35,80	38,44	40,30	45,30	53,22	54,96	55,80
9	Lower leg length (popliteal height)	39,31	2,46	0,12	6,25	33,10	33,40	34,40	39,90	42,81	43,90	44,50
10	Upper hip bone height, sitting	55,74	3,98	0,19	7,14	42,80	45,68	47,79	56,20	61,30	65,70	67,70
11	Lowest rib bone height, sitting	65,56	4,81	0,23	7,34	51,30	54,18	56,29	65,90	72,70	77,00	79,20

Table 7. Anthropometric data for Malaysian males and females, aged 18–24 years (n= 1032) [14]

No.	Measurement (cm)	Mean	SD	SEM	CV (%)	Min	1st	5th	50th	95th	99th	Max
1	Age (year)	19,82	1,07	0,03	5,39	18,00	18,33	19,00	19,00	21,00	23,00	24,00
2	Stature	162,95	8,25	0,26	5,06	141,50	144,87	150,27	163,00	177,14	182,40	186,18
3	Weight (kg)	60,75	14,1	0,44	23,26	36,00	40,00	44,00	58,00	91,00	107,67	120,00
4	Shoulder (biacromial) breadth	40,84	4,04	0,13	9,89	30,20	32,50	34,30	41,10	47,80	50,83	52,80
5	Hip Breadth, sitting	31,52	3,48	0,11	11,04	22,10	22,80	26,60	31,10	38,57	40,80	42,80
6	Shoulder height, sitting	54,30	4,02	0,13	7,40	42,40	43,67	47,20	54,40	60,80	63,57	65,30
7	Elbow height, sitting	19,24	3,23	0,10	16,79	11,40	12,50	14,40	18,90	25,04	27,30	40,50
8	Buttock-popliteal length (seat depth)	47,63	4,01	0,12	8,41	35,80	38,90	40,83	48,00	54,20	55,47	59,40
9	Lower leg length (popliteal height)	40,54	2,20	0,07	5,42	33,10	33,50	35,50	40,80	43,60	44,70	45,70
10	Upper hip bone height, sitting	56,14	3,78	0,12	6,73	42,80	46,10	49,70	56,20	61,74	65,90	76,30
11	Lowest rib bone height, sitting	66,71	4,53	0,14	6,79	51,30	54,60	59,47	66,60	73,84	78,23	89,60

Table 8. Recommended dimensions of lumbar support for motorcyclists based on anthropometric dimensions

Lumbar support design feature	Anthropometric measurement	Design dimension (cm)			Determinant
		Male	Female	Combined	
Bottom of back rest height	Upper hip bone height, sitting	14.7	13.4	14.2	5th of upper hip bone height
Top of back rest height	Lowest rib bone height, sitting	30.8	29.9	30.2	95th of lowest rib bone height
Lumbar support height	Distance between top and bottom of back rest height	16.1	16.5	16.0	Distance between top and bottom of back rest height
Lumbar support width	Hip breadth, sitting	37.6	39.0	38.5	95th of hip breadth, sitting

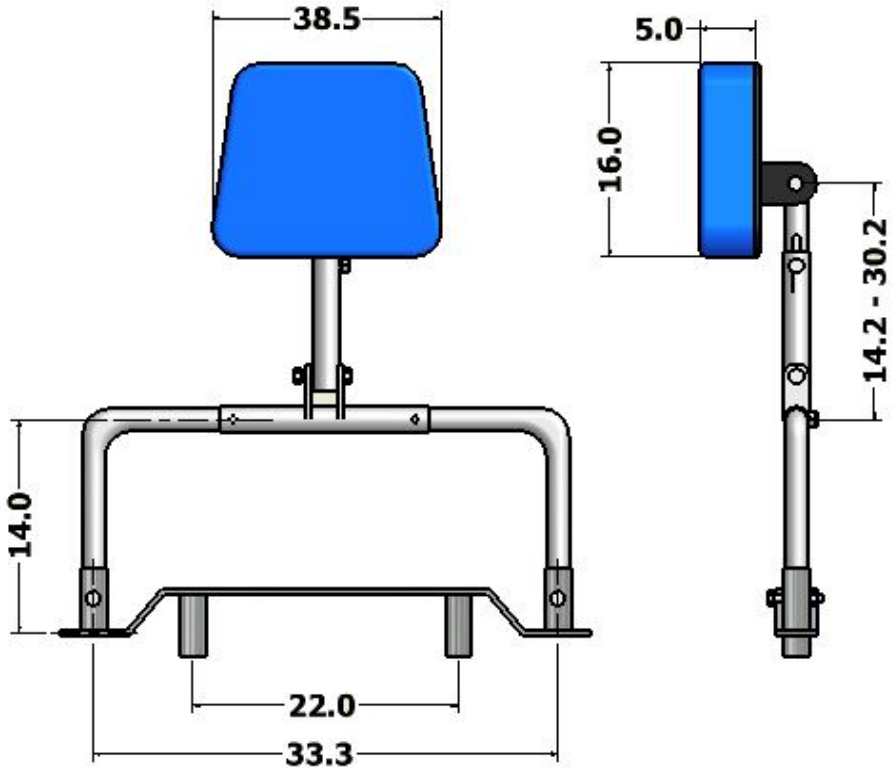


Figure 7. Detail of selected conceptual design (Design 3) (dimension in cm)

The manufacturing process was undertaken in order to construct the proposed prototype of the lumbar support. The process involved three stages: fabrication of the lumbar support frame (Figure 8) and lumbar support cushion (Figure 9) and assembly process (Figure 10).

Figure 8. Fabrication of lumbar support frame

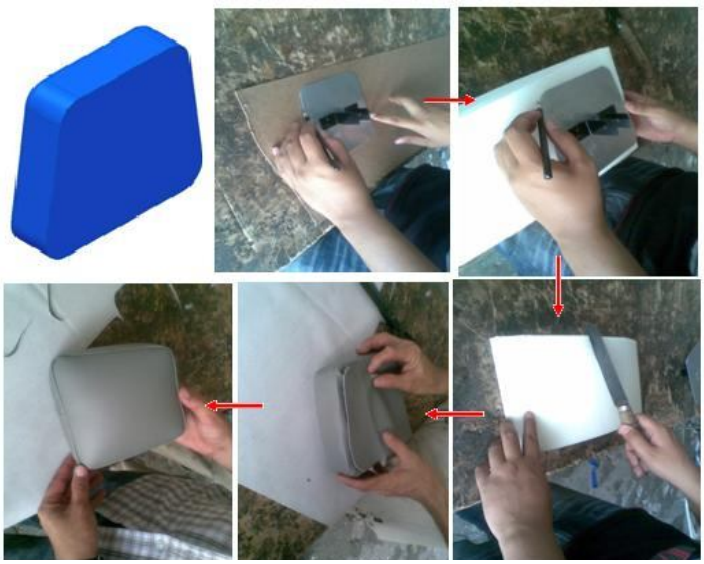


Figure 9. Fabrication of lumbar support cushion

Figure 10. Assembly of lumbar support

The testing of the fabricated lumbar support prototype was conducted. The initial results indicated that the motorcyclists were satisfied with the prototype as it provided comfort to their lumbar region during the riding and reduced the frequency of their posture changes. Furthermore, the lumbar support could be adjusted to suit their lumbar height dimensions. However, further evaluation on the prototype needs to be conducted to determine their stability, solidity, durability and safety over prolonged use.

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Full Paper

Crystal growth and characterisation of a unique trinuclear V^{IV}/V^{V} complex

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Abstract: Single crystals of a mixed-valence trinuclear cluster of formula $[V_2^{V}V^{IV}O_5(C_{12}H_8N_2)_3(SO_4)_2(H_2O)_3]\cdot 6H_2O$ were grown by layer diffusion technique and characterised by single-crystal X-ray diffraction; $P2_1/c$, a = 20.5448(11) Å, b = 11.7647(9) Å, c = 18.1871(9) Å, β = 92.64(0)°, V = 4391.22 (93) Å^3, R = 0.0941 and Rw = 0.1345. A distinct characteristic of the structure is the existence of the rare linear mono-μ-oxo $[V_2^{V}V^{IV}O_5]^{4+}$ building units and the presence of a large number of hydrogen bonds and π-π interactions. The study on the mixed valence state of vanadium by valence bond sum calculations, manganometric titration and cyclic voltammetry, and the presence of π-π interactions by calculation of the harmonic oscillator model of aromaticity indices are presented. The thermogravimetric and differential scanning calorimetric analysis is also reported. The results of UV-Vis spectroscopic study and band gap energy calculation are included.

Keywords: vanadium complex, trinuclear complex, crystal structure, single-crystal X-ray diffraction

INTRODUCTION

Prompted by a variety of valences and coordination chemistry that can be adopted by vanadium and a wide range of potential applications of its complexes [1-3], the interest in vanadium complexes has been unceasing, particularly in those of high nuclearity and mixed valence state. The bi-nuclear complexes containing a mono-μ-oxo $[V_2O_3]^{2+}$ core are thus far the largest class in which the mixed valence state of vanadium is common. Examples of polynuclear V^{IV}/V^{V} complexes with mono-μ-oxo-

vanadium cores are still limited: the tetra-nuclear $[V_40_6(C_2H_5O)_6(C_{12}H_8N_2)_2]$, penta-nuclear $\{[V_2O_4(C_{12}H_8N_2)_2(PO_4)]2VO(OH)\}_{3/4}\{[V_2O_4(C_{12}H_8N_2)_2(HPO_4)]_2\}_{1/4}\cdot 4.5H_2O$ and nona-nuclear $K_7[V_9O_{16}(bdta)_4]\cdot 27H_2O$ (bdta = butanediaminetetraacetate) are known [4-6]. To the best of our knowledge, the first example of the tri-nuclear vanadium complex of this kind with a chemical formula of $[VO_2(phen)(SO_4)(H_2O)]_2(VO(phen)(H_2O)]\cdot 4H_2O$ (*phen* = phenanthroline ligand) was reported by Huang et al. in 2008 [7]. Its hydrothermal synthesis and the novel characteristic of its structure in exhibiting a practically linear $[V_3O_5]^{4+}$ core were reported with a brief description on the EPR and UV-Vis study of the complex.

As a continuation of our interest in the synthesis of new polyoxovanadates using organodiamines of different molecular flexibility and aromaticity, we embark on the synthesis and growing of single crystals of compound $[V_2^{V}V^{IV}O_5(C_{12}H_8N_2)_3(SO_4)_2(H_2O)_3]\cdot 6H_2O$ **(1)**. Although reported earlier [7], the synthesis and crystal growth of **1** by a different route carried out in this study and a detailed description of its crystal structure should be worth reporting. Different ways of determining the mixed valence state of vanadium are presented. The UV-Vis spectroscopic study, cyclic voltammetric analysis and thermorgravimetric-differential scanning calorimetric analysis of **1** were also performed.

MATERIALS AND METHODS

Chemicals

All chemicals were used as-received: 1,10-phenanthroline ($C_{12}H_8N_2$; Fluka, 99%), ethyl alcohol (Merck, 99.9%), ammonium metavanadate (Ajax, 99.5%), sodium hydroxide (Merck, 99%), sulfuric acid (Merck, 95-97%), potassium permanganate (BDS, 99%), sodium sulphite (Ajax, 98%) and potassium bromide (BDH 98.5%).

Crystal Growth and Characterisation

An ethanolic solution of organic ligand (solution A) was prepared by dissolving 0.495 g of 1,10-phenanthroline (*phen*) in 15.0 cm^3 of ethyl alcohol. An aqueous solution of VO_2^+ (solution B) was prepared by dissolving 2.00 g of ammonium metavanadate in 50.0 cm^3 of warm 1.00 mol dm^{-3} sodium hydroxide solution, followed by addition of 80.0 cm^3 of 1.00 mol dm^{-3} sulfuric acid . A portion of solution B (1.50 cm^3) was gently loaded into a glass test tube of 5 mm in diameter and 10 cm in length, followed by 1.50 cm^3 of solution A. Dark green crystals of **1** appeared at the boundary between solutions A and B after leaving for 5 days at atmospheric condition.

The elemental composition of the crystals was semi-quantified using an energy-dispersive X-ray microanalyser equipped with a field-emission scanning electron microscope (JEOL JSM-6335F), whereupon a consistent V:S:O:C:N atomic ratio of 2.8:1.0:3.2:6.7:1.5 (exp.) compared to 2.4:1.0:5.5:6.7:1.3 (calc.) was obtained. A Fourier transform infrared (FTIR) spectrum of the ground crystals as a KBr pellet was collected on a Bruker Tensor 27 FT-IR instrument (4000-400 cm^{-1}, resolution 0.5 cm^{-1}): 3450 cm^{-1}, ν(O–H); 3065 cm^{-1}, ν(aromatic C–H); 1626, 1583, 1519 and 1427 cm^{-1}, ν(aromatic C=C); 1187, 1125 and 1032 cm^{-1}, ν(SO_4); 970 and 937 cm^{-1}, ν(V=O); 870, 848, 778, 736 and 723 cm^{-1}, δ_{oop}(aromatic C-H); 647 and 593 cm^{-1}, δ(V–O–V).

The purity of **1** was assured by X-ray powder diffraction pattern collected on a Bruker D8 Advance diffractometer (Ni filter, Cu *K*α, λ=1.540558 Å, 48 kV, 30 mA). In order to determine the amount of V^{IV} and total $V^{IV,V}$, the aqueous solutions of **1** and its reduced form were titrated against standardised potassium permanganate solution. The solution of the reduced form of **1** was obtained by a reaction with sulphur dioxide generated from sodium sulphite. Cyclic voltammetry (CV) was also conducted using a BAS CV-50W voltammetric analyser (Bioanalytical System, Inc., USA) with Pt (MF-2013, 1.6 mm in diameter), Ag/AgCl (MF-2063) and Pt wire (MW-1032) as working, reference and auxiliary electrodes respectively.

Themogravimetric-differential scanning calorimetric (TG-DSC) analysis was performed using ETZSCH STA 409 PC/PG thermal analyser (Netzsch-Gerätebau GmbH, Germany) (20-1200^{o}C, rate 10^{o}C/min, N_2 gas) to evaluate the thermal stability of **1**. A UV-Vis spectrum of an aqueous solution of **1** from 200 nm to 800 nm was measured with a Perkin Elmer UV LAMDA 25 spectrophotometer. The loss of crystal colour after complete dissolution was noted.

Crystal Structure Determination

Data of 8699 independent reflections were collected in a range of 2.5≤θ/°≤26.1 on a 0.45×0.25×0.15 mm^3 greenish lozenge crystal of **1** using a Stoe IPDS2 diffractometer (Stoe & Cie GmbH, Germany) and Stoe *X*-Area software [8]. A face indexed absorption correction was applied within the Stoe *X*-RED software using Tompa method [9-10]. The data were then reduced and refined resulting in 5365 reflections with $I > 2\sigma(I)$ and internal R of 0.070. The structure was determined by direct method and refined by full-matrix least-square methods using SHELXS97 and SHELXL97 programs via the WinGx program interface [11-13]. The structure was solved and refined in $P2_1/c$, a = 20.5448(11) Å, b=11.7647(9) Å, c = 18.1871(9) Å, β = 92.64(0)°, V = 4391.22 (93) Å^3, Z = 4, R =0.0941 and Rw = 0.1345. The data were of reasonable quality. However, it was not possible to locate hydrogen atoms of the included water molecules. Some disorder in the positions of the water molecules was also detected. Details on data collection and structural deduction and refinement are summarised in Table 1. A rather large deviation of the goodness of fit from unity due to local disorder in the structure of **1** may be noted.

The structure of **1** was first reported by Huang et al. in 2008 [7]. The synthesis by hydrothermal route and the uniqueness of the complex as the first mixed-valence polynuclear vanadium with linear mono-μ-oxo $[V_3O_5]^{4+}$ core were briefly reported. The study of the EPR and electronic spectrum was included. Crystallographic data of the formerly reported structure were compared with the presently reported structure as shown in Table 1, which suggests an approximate equivalence.

Table 1. Crystal data and structure refinement for **1**

	1	Data abstracted from Huang et al. [7]
Formula	$[V_2^{V}V^{IV}O_5(phen)_3(SO_4)_2(H_2O)_3]\cdot 6H_2O$	$[VO_2(phen)(SO_4)(H_2O)]_2[VO(phen)(H_2O)]\cdot 4H_2O$
Formula weight	1117	1091.66
Crystal description	Dark green	*N/A*
Crystal system	Monoclinic	Monoclinic
Space group	$P2_{1/c}$	$P2_{1/c}$
a/ Å	20.5448(11)	20.747(4)
b/ Å	11.7647(9)	11.828(2)
c/ Å	18.1871(9)	18.316(4)
β / °	92.639(4)	93.07(3)
Unit cell volume/ Å^3	4391.2(5)	4488.2(16)
Z	4	4
ρ_{calc}/g.cm^{-3}	1.691	1.616
T/K	150(4)	293(2)
Radiation (λ/Å)	Mo K_α(0.71073)	Mo K_α(0.71073)
Total data collected	24523	37068
R_{int}	0.070	0.0384
Data (I>2σ(I))	8699	10059
Goodness of fit (S)	0.797	1.049
R, R_w (all data)	0.0941, 0.1345	*N/A*
R, R_w(I>2σ(I))	0.055, 0.135	0.0558, 0.1488

RESULTS AND DISCUSSION

Crystal Structure of 1

Compound **1,** $[V_2^{V}V^{IV}O_5(C_{12}H_8N_2)_3(SO_4)_2(H_2O)_3]\cdot 6H_2O$, crystallises in monoclinic space group $P2_1/c$ with cell parameters a = 20.5448(11) Å, b = 11.7647(9) Å, c = 18.1871(9) Å, β = 92.64(0)°, V = 4391.22 (93) Å^3 and Z = 4, which are similar to those of the previously reported structure [7]. Figure 1 shows an asymmetric unit of **1**, depicting three distinct vanadium atoms linked by two μ_2-O11 and μ_2-O12 to form an approximately linear trinulcear vanadium oxide backbone with bond angles being close to linearity: V1-O11-V2 = 163.1° and V(2)-O(12)-V(3) = 159.2°. Selected bond lengths and bond angles in **1** are listed in Table 2 and Table 3 respectively. The terminal V1 and V3 share the common distorted octahedral geometry, coordinated to two N atoms of the chelating *phen* and four O atoms from a monodentate sulphate, a terminal aqua ligand, the vanadyl bond and the oxo-bridge. Similar distorted octahedral geometry is adopted by the mediating V2, coordinated to two N atoms of the chelating *phen*, two *trans* μ_2-O atoms of oxo-bridges, and the vanadyl and sulfate O atoms. The chemical formula of $[V_3O_5(phen)_3(SO_4)_2(H_2O)_3]$ can thus be derived. A common question for polynuclear vanadium complexes is, however, the valence states of the vanadium atoms.

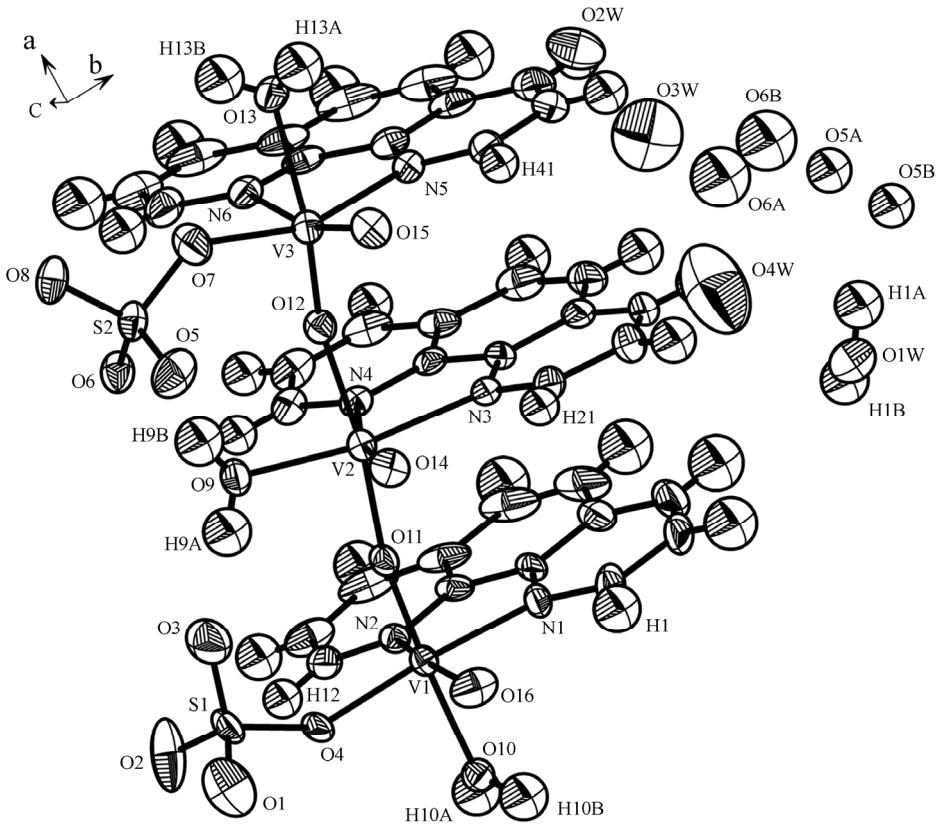

Figure 1. Asymmetric unit of **1** with atomic numbering scheme and drawn with 50% thermal elliptical possibility

Table 2. Selected bond distances (Å) in **1** with standard deviations in brackets

V1—O16	1.600(3)	V3—O12	1.677(3)
V1—O11	1.675(3)	V3—O7	1.930(3)
V1—O4	1.917(3)	V3—O13	2.123(4)
V1—N1	2.132(4)	V3—N5	2.136(4)
V1—O10	2.149(4)	V3—N6	2.296(4)
V1—N2	2.249(3)	S2—O5	1.430(4)
V2—O14	1.592(3)	S2—O8	1.455(3)
V2—O12	1.932(3)	S2—O6	1.468(3)
V2—O11	1.947(3)	S2—O7	1.521(4)
V2—O9	1.998(3)	S1—O2	1.421(4)
V2—N3	2.124(4)	S1—O1	1.457(4)
V2—N4	2.307(3)	S1—O3	1.462(5)
V3—O15	1.596(3)	S1—O4	1.510(3)

Table 3. Selected bond angles (°) in **1** with standard deviations in brackets

O16—V1—O11	104.73(16)	O12-V2-N3	87.57(13)	N5-V3-N6	73.96(13)
O16—V1—O4	104.79(18)	O11-V2-N3	96.01(13)	O5-S2-O8	113.5(2)
O11—V1—O4	99.86(14)	O9-V2-N3	159.64(14)	O5-S2-O6	111.2(2)
O16—V1—N1	92.14(18)	O14-V2-N4	166.12(15)	O8-S2-O6	110.40(19)
O11—V1—N1	88.54(15)	O12-V2-N4	80.55(12)	O5-S2-O7	109.1(2)
O4 —V1—N1	158.32(14)	O11-V2-N4	82.81(12)	O8-S2-O7	103.9(2)
O16—V1—O10	87.57(16)	O9-V2-N4	86.43(14)	O6-S2-O7	108.3(2)
O11—V1—O10	165.39(13)	N3-V2-N4	73.57(13)	O2-S1-O1	109.3(3)
O4 —V1—O10	84.22(13)	O15-V3-O12	104.37(16)	O2-S1-O3	116.4(3)
N1 —V1—O10	83.05(14)	O15-V3-O7	104.52(17)	O1-S1-O3	105.5(3)
O16—V1—N2	161.26(17)	O12-V3-O7	97.97(15)	O2-S1-O4	106.8(2)
O11—V1—N2	88.14(14)	O15-V3-O13	93.89(15)	O1-S1-O4	107.2(2)
O4 —V1—N2	85.91(13)	O12-V3-O13	161.30(13)	O3-S1-O4	111.2(2)
N1 —V1—N2	74.35(14)	O7-V3-O13	81.01(15)	S1-O4-V1	140.7(2)
O10-V1-N2	78.10(13)	O15-V3-N5	89.66(15)	S2-O7-V3	137.3(2)
O14-V2-O12	99.86(15)	O12-V3-N5	95.24(14)	V1-O11-V2	163.10(18)
O14-V2-O11	98.38(15)	O7-V3-N5	157.48(16)	V3-O12-V2	159.23(18)
O12-V2-O11	161.23(12)	O13-V3-N5	80.64(14)		
O14-V2-O9	107.45(16)	O15-V3-N6	162.33(16)		
O12-V2-O9	85.36(14)	O12-V3-N6	84.12(14)		
O11-V2-O9	85.00(13)	O7-V3-N6	89.31(16)		
O14-V2-N3	92.56(15)	O13-V3-N6	77.20(13)		

The bond valence sum (BVS) calculation was attempted using the refined bond lengths listed in Table 2 with the following assumed parameters: $R_0(V^{V,IV}–O^{-II})$ = 1.735, $R_0(V^{V}–O^{-II})$ = 1.803, $R_0(V^{IV}–O^{-II})$ = 1.780, $R_0(V^{V,IV}–N^{-III})$ = 1.875, and b = 0.370 [14]. The BVS of 4.81, 4.15 and 4.85 were obtained for V1, V2 and V3 respectively, indicating the presence of the expected V^{IV} and V^{V} with twice the amount of the latter. The result of manganometric titration confirms the calculation: 0.915 mol of V^{IV} per formula unit. The CV analysis confirms this by the presence of an irreducible reduction peak of V^{V} to V^{IV} at +0.242 V vs Ag/AgCl with a maximum current of 4.48 A. Based on the BVS calculation, manganometric titration and the CV experiment, a mixed $2V^{V}$:V^{IV} valence state for vanadium and the precise formula of $[V_2{}^{V}V^{IV}O_5(C_{12}H_8N_2)_3(SO_4)_2(H_2O)_3]$ can thus be deduced. This leads to the conclusion on charge neutrality for the cluster, which confirms the presence of only water molecules as the extra-cluster species and justifies the assignment of the extra-cluster O atoms as water during the crystal structure deduction and refinements. The chemical formula with six extra-cluster water molecules, viz. $[V_2{}^{V}V^{IV}O_5(C_{12}H_8N_2)_3(SO_4)_2(H_2O)_3]\cdot 6H_2O$, is then established.

The phenanthroline (*phen*) ligands are located on the same side of the vanadium oxide backbone with the distances of 3.689 Å and 3.670 Å between the centroids of two adjacent molecular planes as shown in Figure 2. These distances are in a range possible for the π-π interactions to occur [15-17], which can be regarded as an important parameter regulating the spatial arrangement of these chelating ligands. It is apparent that these phenanthroline ligands are not exactly parallel to each other but slightly converge towards the free end of the molecule. Figure 3 depicts the centroids of the central benzene rings of the *phen* ligands, showing the panning angles of 9.48° and 21.80° for the superjacent and subjacent ligands from the middle one. The relative arrangement of these organic ligands indicates that other interactions are present that subjugate the weak π-π interactions, which favour the superimposed position of the ligands.

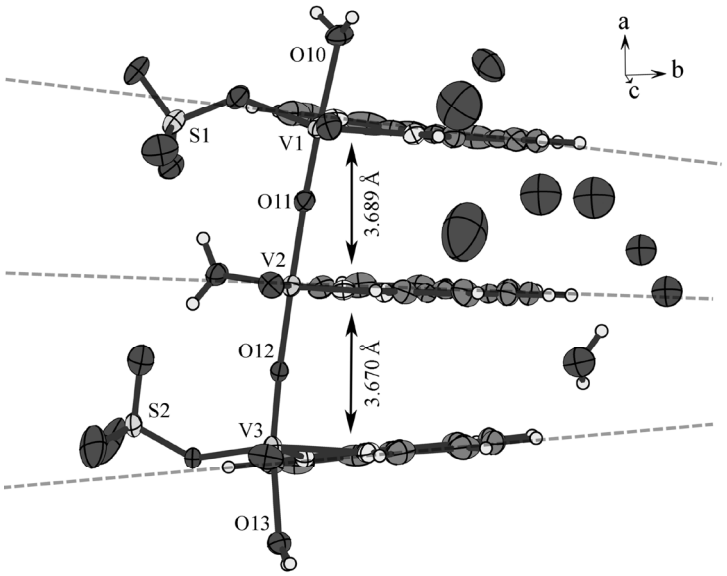

Figure 2. Illustration showing the relative spatial arrangement of *phen* and the corresponding inter-molecular planar distances

The interaction between π electrons commonly encountered in the stacking of aromatic molecules should impart an influence on their aromaticity. Here, the harmonic oscillator model of aromaticity (HOMA) index was used to evaluate the presence of this interaction [18-20]. According to a survey of structures consisting of *phen* in the molecules both in coordination and non-coordination modes found in the Cambridge structural databases [21], it is evident that the values of HOMA indices are distributed in different ranges depending on both the coordination and the π-π interaction. For non-coordinated *phen*, the average indices of three fused benzene rings are distributed in a range of 0.25-0.50 for those without π-π interactions and 0.66-0.83 for those with the interactions. On the other hand, the presence of π-π interactions seems to be common with coordination with average indices distributed in a higher range of 0.70-0.94, suggesting the preference of these aromatic ligands to arrange themselves in such a way as to maximise the interactions. In the structure of **1**, the HOMA indices were calculated at similar values of ca. 0.80 for each *phen* ligand. This is in very good

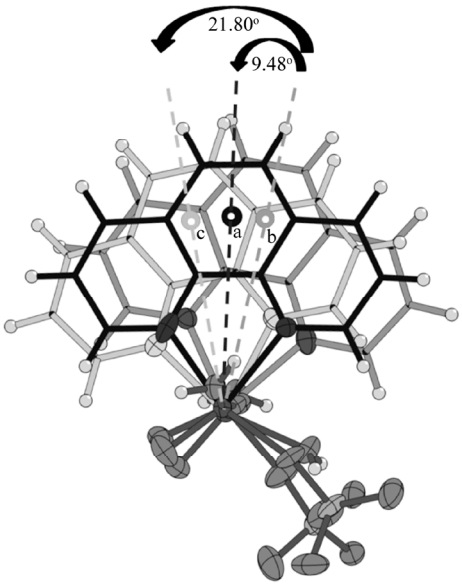

Figure 3. Illustration showing relative locations of the centroids (*a*, *b* and *c*) of the central benzene rings of the superjacent, middle and subjacent *phen* ligands respectively, with relative panning angles of a and c from b

agreement with the surveyed HOMA indices for those complexes with π-π interacting *phen* ligands, hence the suggested presence of such weak interaction in **1**.

The analysis for hydrogen bonding according to the definition proposed by Jeffrey [22] was performed on the crystal structure and revealed a large number of hydrogen bonding interactions as depicted in Figure 4 and listed in Table 4. The weak C-H···O hydrogen bonds formed between *phen* and the neighbouring O atoms of both ligated and free water molecules as well as the nearby sulphate group may account for the subjugation of the π-π interactions and the consequent orientation of these organic ligands. The strong hydrogen bonds of O-H···O type between the ligated water of the mediated V2 and the nearby sulphates also provide explanation for the orientation of the pending SO_3 motifs of V1 and V3 that incline towards each other with the bending angles of 140.71° and 137.32° for V1-O3-S1 and V3-O7-S2 respectively.

Compared to the complex, $[VO_2(phen)(SO_4)(H_2O)]_2(VO(phen)(H_2O)]\cdot 4H_2O$, reported by Huang et al. [7], structure **1** contains two more molecules of water of crystallisation and therefore a larger number of hydrogen bonding interactions. This may stem from the difference in crystal growth technique and condition. Crystals of **1** were grown at ambient temperature and pressure, whereas those of the former were obtained hydrothermally. The larger number of water outside the coordination sphere, however, does not significantly affect the solid state registry of the compound.

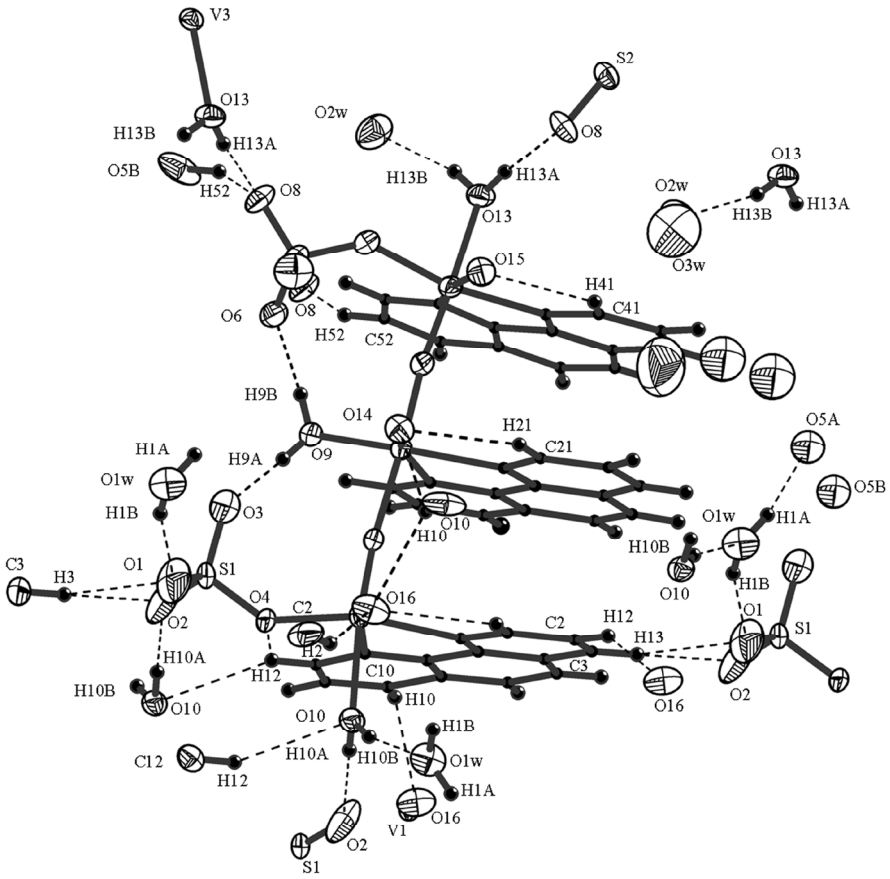

Figure 4. Hydrogen bonding interactions (dotted lines) of different types as listed in Table 4

UV-Vis Spectroscopic Study

The UV-Vis spectrum measured on the aqueous solution of **1** at a concentration of $5x10^{-4}$ mol dm^{-3} (Figure 5) exhibits absorption only in the UV region. The absorption bands characteristic of *phen* are clearly present with the maxima at 230 nm (3,205 $dm^3mol^{-1}cm^{-1}$) and 264 nm (2,150 dm^3mol^{-1} cm^{-1}), both of which are attributable to the intraligand π-π* transitions. The most intense band at 206 nm (10,584 $dm^3mol^{-1}cm^{-1}$) can be assigned to the LMCT process of the terminal oxygen while a broad band appearing as shoulder at 270 nm is the characteristic band associated to the LMCT of the bridging oxygen [23-25]. The absorption coefficients (α) can be calculated from the maximum (λ_{max}) of each band, and the plot between $(\alpha h\nu)^2$ and $h\nu$ can be made (inset of Figure 5) if only the direct electronic transition is assumed [26]. The band gap energy for each transition can be obtained by extrapolation of the positive tangent line for each $h\nu$ to α=0: 4.3017, 5.2317 and 5.8182 eV for the transitions with λ_{max} at 264, 230 and 206 nm respectively.

Table 4. Summary of hydrogen bonding geometry for **1** (Standard deviations are in brackets)

D	A	H···A (Å)	D···A (Å)	∠ D-H···A (°)
$O1_W$	$O5_A$	2.17(5)	2.981(7)	158(5)
$O1_W$	O1	1.99(4)	2.807(5)	161(6)
O9	O3	1.72(4)	2.560(5)	175(7)
O9	O6	1.76(4)	2.604(5)	176(5)
O10	O2	1.91(4)	2.750(5)	179(7)
O10	$O1_W$	1.87(5)	2.690(5)	164(5)
O13	O8	1.82(5)	2.656(5)	174(5)
O13	$O2_W$	1.93(5)	2.728(5)	163(4)
C1	O16	2.57	2.994(7)	108
C2	O16	2.46	3.308(7)	149
C3	O1	2.50	3.270(7)	138
C3	O2	2.35	3.272(8)	163
C10	O14	2.56	3.243(7)	129
C10	O16	2.49	3.212(7)	133
C12	O4	2.56	3.058(5)	113
C12	O10	2.57	3.365(6)	142
C21	O14	2.49	2.957(6)	110
C41	O15	2.43	2.894(6)	110
C52	O8	2.45	3.246(7)	142

It is intriguing that there was no absorption in the visible light region (325-800 nm) for the solution of **1**, which was also colourless. This might be due to the loss of long-range order of the solid state structure when it was made into solution. It has been reported, however, that a broad absorption band which should be evidence for the inter-valence charge transfer between V^{IV} and V^{V} was observed in this region for the spectrum collected on a solid sample [7].

Thermogravimetric and Differential Scanning Calorimetric Analysis

On heating **1** under the flow of N_2 gas, four stages of endothermic weight loss were observed (Figure 6). The first weight loss of 9.86% occurring between 80-150°C agrees well with the weight of six non-coordinating water molecules (9.58%), while the second loss of 5.14% observed at 150-340°C is approximately equivalent to the weight of three coordinating water molecules (4.79%). The corresponding endothermic features found in the DSC curve reflect the influence of the hydrogen bonding interactions involved with these water molecules. The next two subsequent weight losses found from 340°C to 700°C totalled 45.00%, which corresponds well with the release of three coordinated *phen* ligands. It should be noted that the argument is made based only on the agreement of weight percentages; further experiments on identification of the liberated species have to be performed if a definite conclusion is to be obtained.

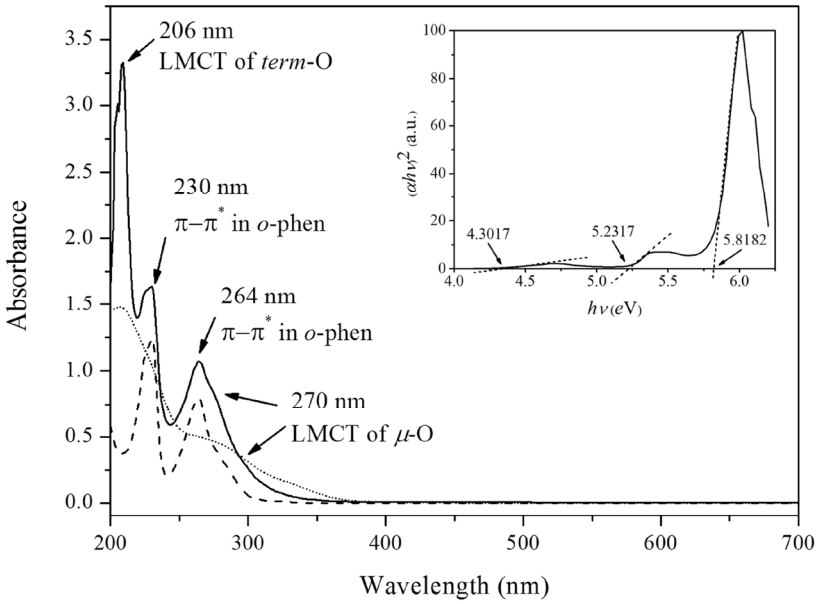

Figure 5. The UV-Vis spectrum of aqueous solution of **1** ($5x10^{-4}$ $moldm^{-3}$, solid line) compared with those of the ligand (dash line) and vanadium precursor (dotted line). The plot between $(\alpha h\nu)^2$ and hv is provided in the inset

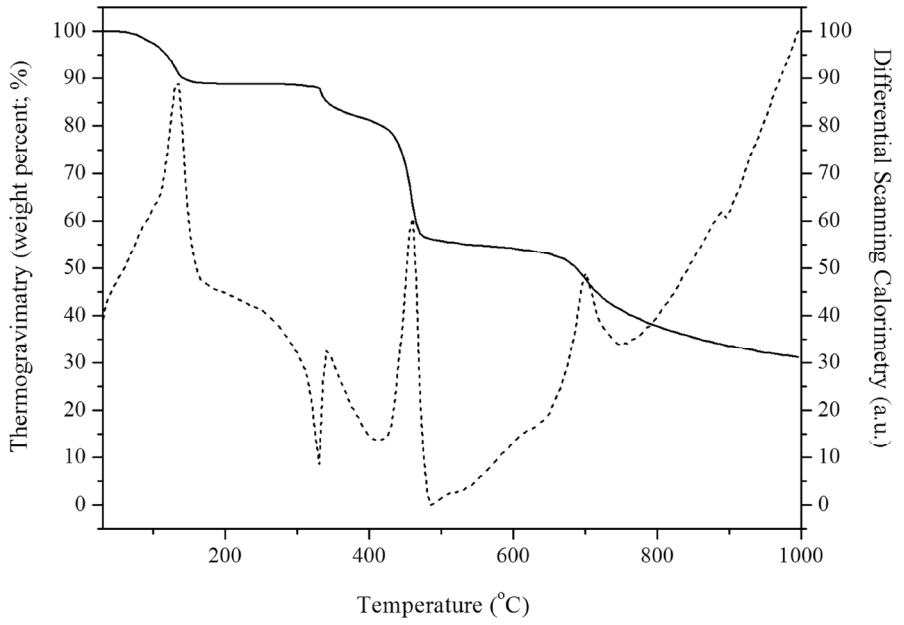

Figure 6. Thermogravimetric (solid line) and differential scanning calorimetric (dash line) graphs collected on **1**

CONCLUSIONS

A rare example of mixed-valence trinuclear cluster, $[V_2^{V}V^{IV}O_5(C_{12}H_8N_2)_3(SO_4)_2(H_2O)_3]\cdot 6H_2O$, has been synthesised at ambient atmosphere. Its crystal structure has been determined and refined with excellent agreement with the previously reported structure which had fewer water of crystallisation. Detailed analysis of the structure indicates the presence of both π-π interactions and a large number of intra- and inter-cluster hydrogen bonds, which impart influence on spatial orientation of the cluster-building motifs. According to the bond valence sum calculations, the manganometric titration and cyclic voltammetry, the mixed valence of V^{IV} and V^{V} in the ratio of 1:2 can be deduced. The presence of six molecules of water of crystallisation is consistent with the results of the thermogravimetric/differential scanning calorimetric analysis, which suggest sequential loss of water of crystallisation, ligated water and the phenanthroline ligands.

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SUPPLEMENTARY MATERIAL

Crystallographic data for compound **1** is available as supplementary material of this article in cif format [download].

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Full Paper

Investigating the performance of single- and multichannel wireless receivers in generic-K fading channels

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Abstract: By using versatile generic-K statistical model, a performance analysis of wireless system has been carried out through the composite fading channel scenarios. The composite fading model used here is flexible enough to represent all forms of mixed shadowed-fading channel conditions. With the aid of moment generating function (MGF) approach and Padé approximation (PA) technique, different performance measures such as outage probability and average bit error rate (ABER) have been evaluated for a variety of digital modulation formats. In contrast to previously obtained relatively complicated expressions in terms of MeijerG & Whittaker special functions, the PA technique has been used here to find tractable rational expressions for the MGF of output SNR. Using these simple rational expressions, the performance evaluations have been done for both single- and multichannel receivers under different shadowed-fading channel conditions. The numerical results are also validated through computer simulations, which show a perfect match.

Keywords: shadowed fading channel, outage probability, average bit error rate, moment generating function, diversity reception, computational mathematics, fading channel modeling

INTRODUCTION

Wireless communication channels are impaired by the detrimental effects such as multipath fading and shadowing. Considerable efforts have been devoted to statistically model these effects. The constructive and destructive combinations of delayed, reflected, scattered and diffracted multipath signal components lead to multipath fading. Various multipath fading models were used in

the past by considering different radio propagation environments and underlying communication scenarios [1a]. Based on various indoor and outdoor empirical measurements, there was a general consensus that shadowing is to be modelled using lognormal distribution [2-4]. Usually, multipath fading models assume constant average signal power, whereas it becomes random in some situations such as congested downtown areas with slow moving pedestrians and vehicles [5] and land-mobile satellite systems subject to urban or vegetative fading [6-7]. This type of mixed fading situation is referred to as composite fading or shadowed fading. Different combinations of fading-shadowing distribution have been used in the literature to model composite fading. However, much work is devoted to Rayleigh-lognormal and Nakagami-lognormal combinations [2, 4-5, 8-9]. The main drawback of these composite fading models is their complicated mathematical form due to the use of lognormal distribution for shadowing. Consequently, analytical performance evaluation becomes difficult. For analytical simplification, K-distribution, which uses gamma distribution in place of lognormal distribution, has been used as an alternative to Rayleigh-lognormal [10-12]. In the past, K-distribution was also used for radar applications [13-14]. However, it lacks flexibility and so cannot be used to fit diverse shadowed fading scenarios. A relatively new generic-K composite distribution has been proposed [15-16], which is flexible enough to create most of shadowed fading conditions observed in current wireless communication systems. By applying this model, performance analysis of single- and multichannel wireless receivers has been carried out in terms of Whittaker and MeijerG special functions [17-19]. The closed-form expressions derived therein based on these special functions, however, suffer from a major drawback despite being the first of their kind in the literature and having an elegant form. Although these special functions can be self-evaluated using modern symbolic mathematical packages (Mathematica & Maple), they fail to handle integrals involving such functions [1b]. Especially, the higher values of shaping parameters k & m lead to numerical instabilities and erroneous results. This renders the expressions impractical from the perspective of ease of computation. Thus, it is desirable to find alternative closed-form expressions for the moment generating function (MGF) of the generic-K random variable that are simple to evaluate and at the same time suitable for higher values of parameters k & m.

In this paper, Padé approximation (PA) is used to obtain simple-to-evaluate rational expressions for the MGF of generic-K random variable. These expressions are used to evaluate the average bit error rate (ABER) performance of important digital modulation schemes of both single- and multichannel receivers employing maximal ratio combining (MRC). Performance evaluation is done through numerous shadowed fading scenarios represented by different values of parameters k & m. The outage probability analysis of MRC is also performed. Earlier, the PA has been used effectively for performance analysis in generalised-Gamma, Nakagami-m and Weibull fading channels [20-22]. However, the computationally efficient and unified performance analysis with and without MRC diversity operating over generic-K fading is not available in the open literature and thus is the topic of this contribution.

STSTEM AND CHANNEL MODEL

Signal transmission over slow, frequency-nonselective generic-K shadowed fading channel is assumed. The baseband representation of the received signal is given by $y = sx + n$, where s is the transmitted baseband symbol which can take different values from modulation alphabets such as M-ary quadrature amplitude modulation (MQAM) and M-ary phase shift keying (MPSK), x is the channel shadowed-fading envelope which is generic-K distributed, and n is the additive white Gaussian noise (AWGN). The probability density function (PDF) [15] of the generic-K random variable is given by

$$f_x(x) = \frac{4m^{(k+m)/2} x^{(k+m-1)}}{\Gamma(m)\Gamma(k)\Omega^{(k+m)/2}} K_{k-m}\left\{2x\left(\sqrt{\frac{m}{\Omega}}\right)\right\} \qquad x \geq 0 \tag{1}$$

where k and m are the distribution shaping parameters, $K_{k-m}\{.\}$ is the modified Bessel function of second kind and order '*k-m*', $\Gamma(.)$ is the Gamma function and Ω is the average fading power such that $\Omega = \frac{E[x^2]}{k}$ [15], where $E[.]$ denotes expectation. For wireless systems, (1) provides a versatile and simple way to model all forms of fading conditions including shadowing. By varying the two shape parameters k and m, different levels of fading and shadowing can be described. When m=1, (1) characterises Rayleigh-lognormal or K-distribution fading; higher values of m correspond to Shadowed-Rician fading channels [16]. For $k \to \infty$, shadowing is absent and it approximates the Nakagami-m fading. However, values of k in the range of 6-8 are sufficient to make the channel solely dependent on m [16]. Low values of k and m correspond to severe fading and shadowing, but as both k and $m \to \infty$, (1) describes ideal channel condition (AWGN). It is well known that the performance of any communication system in terms of ABER and signal outage depends on the statistics of the signal-to-noise ratio (SNR). The instantaneous SNR per received symbol is $\gamma = \frac{x^2 E_b}{N_0}$ and the average SNR is $\overline{\gamma} = \frac{E[x^2] E_b}{N_0}$, where E_b is the average signal energy per bit [1a] and N_0 represents single-sided power spectral density of the AWGN. From the random variable transformation [1a], the PDF of instantaneously received SNR can be given by

$$f_\gamma(\gamma) = 2\left(\frac{km}{\overline{\gamma}}\right)^{\frac{(k+m)}{2}} \frac{\gamma^{(k+m-2)/2}}{\Gamma(m)\Gamma(k)} K_{k-m}\left\{2\left(\sqrt{\frac{km\gamma}{\overline{\gamma}}}\right)\right\} \tag{2}$$

Using (2), the n^{th} moment of γ can be found in closed form as

$$E[\gamma^n] = \left(\frac{\overline{\gamma}}{km}\right)^n \frac{\Gamma(k+n)\Gamma(m+n)}{\Gamma(m)\Gamma(k)} \tag{3}$$

In order to quantify the performance in terms of ABER and signal outage, the well-known MGF approach [1c] is used. Based on this approach, it is required to find alternative closed-form expressions for the MGF which are simpler to compute and valid for higher values of fading parameters. Towards that end, PA is used as described below.

The MGF of random variable $\gamma > 0$ is given by

$$M_\gamma(s) = E\left[e^{-sx}\right] = \int_0^\infty e^{-s\gamma} f_\gamma(\gamma)\, d\gamma \tag{4}$$

Using the n^{th} moment of the instantaneous SNR statistics available in closed form given by (3) and Taylor series expansion of $e^{-s\gamma}$, the MGF given by (4) can be expressed in terms of a power series as

$$\begin{aligned} M_\gamma(s) &= \sum_{n=0}^{\infty} \frac{(-1)^n}{n!} E\left(\gamma^n\right).s^n \\ &= \sum_{n=0}^{\infty} c_n s^n \end{aligned} \tag{5}$$

where $c_n = \frac{(-1)^n}{n!}\left(\frac{\overline{\gamma}}{km}\right)^n \frac{\Gamma(k+n)\Gamma(m+n)}{\Gamma(m)\Gamma(k)}$. The infinite series in (5) is not guaranteed to converge for all values of s, but it is possible using PA to obtain efficiently the limiting behaviour of a power series in compact rational function form [23-24]. In particular, the one-point PA of order $(D-1/D)$ is defined from the series (5) in a rational form by

$$M_\gamma(s) \cong \frac{\sum_{i=0}^{D-1} a_i s^i}{\sum_{j=0}^{D} b_j s^j} \tag{5}$$

where a_i and b_j are the coefficients such that

$$\frac{\sum_{i=0}^{D-1} a_i s^i}{\sum_{j=0}^{D} b_j s^j} = \sum_{n=0}^{2D-1} c_n s^n + O\left(s^{2D}\right) \tag{6}$$

where $O\left(s^{2D}\right)$ represents the terms of order higher than 2*D*-1. The coefficients b_j can be found using (assuming $b_0 = 1$) the following equations:

$$\sum_{j=0}^{D} b_j c_{D-1-j+l} = 0 \qquad 0 \le l \le D \tag{7}$$

The above equations form a system of *D* linear equations having *D* unknown denominator coefficients in (6). This system of equations can be uniquely solved as long as the determinant of its Hankel matrix is non-zero [23]. The choice of the value of *D* is indeed a critical issue as it represents

a trade-off between the accuracy of the PA and the complexity of the system of equations to be solved. After solving for the values of b_j, the set a_i can now be obtained from

$$a_i = c_i + \sum_{p=1}^{min(D,i)} b_i c_{i-p} = 0 \qquad 0 \le i \le D-1 \tag{8}$$

Having obtained the coefficients of denominator and numerator polynomials, an appropriate expression for the MGF of the output SNR is now available in rational function form. We are now ready to present two of the most important performance measures, namely the outage probability and the ABER for different modulation schemes.

PERFORMANCE ANALYSIS

In this section, the performance of various classes of receivers operating over generic-K shadowed fading channel is presented in terms of ABER and outage probability.

Averge Bit Error Rate (ABER)

M-ary quadrature amplitude modulation (MQAM)

In the single channel receiver, using alternative Gaussian-Q function form, the conditional bit error rate of Gray encoded MQAM [25] is given as

$$P_b(\gamma_b) = \frac{4\left(\sqrt{M}-1\right)}{\pi\sqrt{M}\,log_2(M)} \sum_{i=0}^{\sqrt{M}/2-1} \int_0^{\pi/2} exp\left(-\frac{(2i+1)^2}{2sin^2\phi}\frac{3log_2(M)}{(M-1)}\gamma_b\right)d\phi \tag{9}$$

where γ_b is the instantaneous SNR per bit. Averaging over the PDF of the received SNR, the ABER becomes

$$\overline{P}_b = \frac{4\left(\sqrt{M}-1\right)}{\pi\sqrt{M}\,log_2(M)} \sum_{i=0}^{\sqrt{M}/2-1} \int_0^{\pi/2} M_\gamma\left(\frac{(2i+1)^2}{2sin^2\phi}\frac{3log_2(M)}{(M-1)}\right)d\phi \tag{10}$$

where $M_\gamma(.)$ is the MGF of generic-K distributed random variable. In the case of MRC receiver, the total received output SNR is equal to the sum of SNR of the independent channels. For L independent and identical channels, the MGF of the output SNR is expressed as the product of the MGF associated with each channel [1c]. Thus, ABER of the MRC receiver is given by

$$\overline{P}_{MRC} = \frac{4\left(\sqrt{M}-1\right)}{\pi\sqrt{M}\,log_2(M)} \sum_{i=0}^{\sqrt{M}/2-1} \int_0^{\pi/2} M_\gamma\left(\frac{(2i+1)}{2sin^2\phi}\frac{3log_2(M)}{(M-1)}\right)^L d\phi \tag{11}$$

M-ary phase shift keying (MPSK)

In the single channel receiver, using alternative Gaussian-Q function form, the conditional bit error rate of Gray encoded MPSK [25] is given as

$$P_b(\gamma_b) \cong \frac{2}{\pi \max\left(\log_2(M),2\right)} \sum_{i=1}^{\max(M/4,1)} \int_0^{\pi/2} exp\left(-sin^2\frac{(2i-1)\pi}{M}\frac{\log_2(M)}{sin^2\phi}\gamma_b\right)d\phi \tag{12}$$

Averaging over the PDF of the received SNR, the ABER becomes

$$\overline{P}_b \cong \frac{2}{\pi \max\left(\log_2(M),2\right)} \sum_{i=1}^{\max(M/4,1)} \int_0^{\pi/2} M_\gamma\left(sin^2\frac{(2i-1)\pi}{M}\frac{\log_2(M)}{sin^2\phi}\right)d\phi \tag{13}$$

For the MPSK receiver employing MRC, the ABER is given by

$$\overline{P}_{MRC} \cong \frac{2}{\pi \max\left(\log_2(M),2\right)} \sum_{i=1}^{\max(M/4,1)} \int_0^{\pi/2} M_\gamma\left(sin^2\frac{(2i-1)\pi}{M}\frac{\log_2(M)}{sin^2\phi}\right)^L d\phi \tag{14}$$

Binary differential phase shift keying (BDPSK)

For the single channel receiver employing BDPSK, the conditional bit error rate is given by [1c]

$$P_b(\gamma_b) = 0.5\,exp(\gamma_b) \tag{15}$$

The corresponding ABER in the case of single and MRC receivers will be given respectively as

$$\begin{aligned} \overline{P}_b &= 0.5M_\gamma(1) \text{ and} \\ \overline{P}_{MRC} &= 0.5\left(M_\gamma(1)\right)^L \end{aligned} \tag{16}$$

Outage Probability

The signal outage probability is defined as the probability that the instantaneous SNR falls below a certain threshold, γ_{th}, i.e.

$$P_{out}(\gamma_{th}) = P(SNR < \gamma_{th}) \tag{17}$$

For the single channel receiver, using MGF approach [1d], the outage probability can be computed as

$$P_{out}(\gamma_{th}) = \frac{1}{2\pi j}\int_{\varepsilon-j\infty}^{\varepsilon+j\infty} \frac{M_\gamma(s)}{s} e^{s\gamma_{th}} ds \tag{18}$$

In the case of the MRC receiver with L identical and independently distributed channels, the signal outage probability can be given by

$$P_{MRC\_out}(\gamma_{th}) = \frac{1}{2\pi j}\int_{\varepsilon-j\infty}^{\varepsilon+j\infty} \frac{\left[M_\gamma(s)\right]^L}{s} e^{s\gamma_{th}} ds \tag{19}$$

where $M_\gamma(s)$ is the MGF of the output SNR random variable and ε is a properly chosen constant in the region of convergence of complex s-plane. Interestingly, since $M_\gamma(s)$ is given in rational form, one can use the partial fraction expansion of $\left[M_\gamma(s)\right]/s$ in (19) or $\left[M_\gamma(s)\right]^L/s$ in (20) to evaluate the outage probability as

$$\begin{aligned} P_{MRC\_out}(\gamma_{th}) &= \frac{1}{2\pi j}\int_{\varepsilon-j\infty}^{\varepsilon+j\infty}\sum_{i=1}^{N_p}\frac{\lambda_i}{s+p_i}e^{s\gamma_{th}}ds \\ &= \frac{1}{2\pi j}\sum_{i=1}^{N_p}\int_{\varepsilon-j\infty}^{\varepsilon+j\infty}\frac{\lambda_i}{s+p_i}e^{s\gamma_{th}}ds \\ &= \sum_{i=1}^{N_p}\lambda_i e^{-p_i\gamma_{th}} \end{aligned} \tag{20}$$

where p_i are the N_p poles of rational function in s with λ_i its residues. Each term inside the summation in (21) represents a simple rational function form. Clearly, using the rational approximation for the MGF provided by the PA, all the integrals in (11), (12), (14), and (15) can be easily evaluated numerically and are found to be very stable. Moreover, the closed-form expressions can also be found for the integrals given in (19) and (20) using the inverse Laplace transform of a rational function.

NUMERICAL AND SIMULATION RESULTS

Performance evaluation results are presented here based on the analytical framework developed in the previous section. Numerical results have been obtained (using D = 7) with acceptable accuracy for the target error rate of 10^{-4}. However, it is always possible to choose a higher value of D to enhance the accuracy as long as the Hankel matrix is not rank deficient. Table 1 lists the $\{a_i\}$ and $\{b_j\}$ sets for the rational function form of MGF for various shadowed fading conditions using different values of m and k. The values of fading parameters were decisively selected to represent standard shadowed fading conditions. The ABER of digital modulations and outage probability through single- and multichannel receivers were numerically evaluated using simple rational expressions and compared for accuracy with simulation results.

Table 1. Numerator and denominator coefficients of rational expressions of MGF

m	k	Representative channel condition	Numerator coefficients $\{a_i\}$ (a_0=1)	Denominator coefficients $\{b_j\}$ (b_0=1)
1	1	*Severe*	{48,835,6560,23544,33984,13068}	{49,882,7350,29400,52920,35280, 5040}
1	4	*Shadowed-Rayleigh*	{33/2,815/8,2365/8,6615/16,8085/32,12495/256}	{35/2,945/8,1575/4,11025/16,19845/32,33075/128,4725/128}
3.5	8	*Nakagami-m*	{-7.4,-25.5,23.9,-6.8,0.11,-0.19e-2}	{-6.43,-32.66,-51.4,-37.95,-13.96,-2.39,0.14}
10	5	*Shadowed-Rician*	{1.96,1.24,0.24,-6.8e-3,1.8e-4,-3.2e-6}	{2.96,3.54,2.19,0.75,0.14,1.3e-3,4.7e-4}
20	8	*Approached ideal*	{0.95,0.15,-0.018,0.0011,-4.4e-5,9.35e-7}	{1.95,1.51,0.61,0.14,0.019,0.14e-2,0.42e-4}

ABER of M-ary Modulations

Here, three illustrative examples for performance evaluation of the wireless receiver in terms of ABER were selected. The first is depicted in Figure 1 for the case of 16-QAM, the second in Figure 2 for the case of 16-PSK and the third in Figure 3 for the case of BDPSK, all three versus the average SNR per bit. Computer simulation results of ABER for the three representative channel conditions (m = 1, k = 4; m = 3.5, k = 8; and m = 20, k = 8) was obtained and compared with numerical results evaluated using PA technique for similar channel conditions. As can be seen, simulation results corroborate well the numerical results, which gives clear-cut evidence for accuracy in the derived expressions. In Figures 1-3, both single and dual MRC wireless systems are considered. It is evident from the figures that the ABER improves as average SNR per bit ($\overline{\gamma}_b$) increases and, also, for a fixed value of $\overline{\gamma}_b$, ABER improves with increase in k and/or m. As expected, ABER performance of dual MRC is better than that of single channel system in all channel conditions for a fixed value of $\overline{\gamma}_b$.

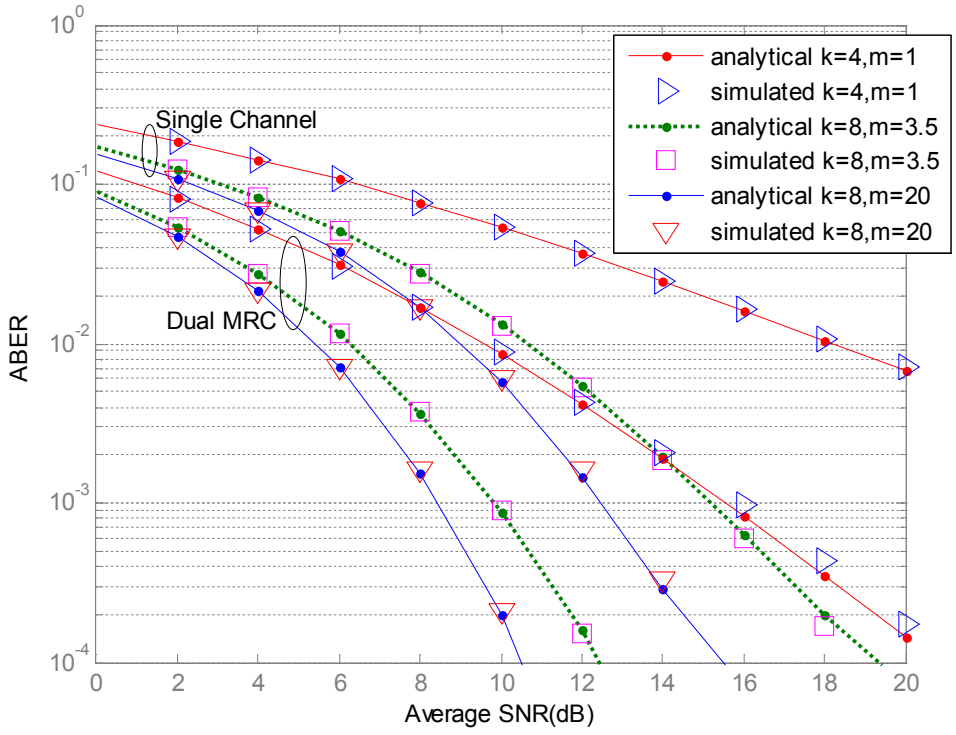

Figure 1. ABER of 16-QAM versus average SNR per bit of representative channel conditions

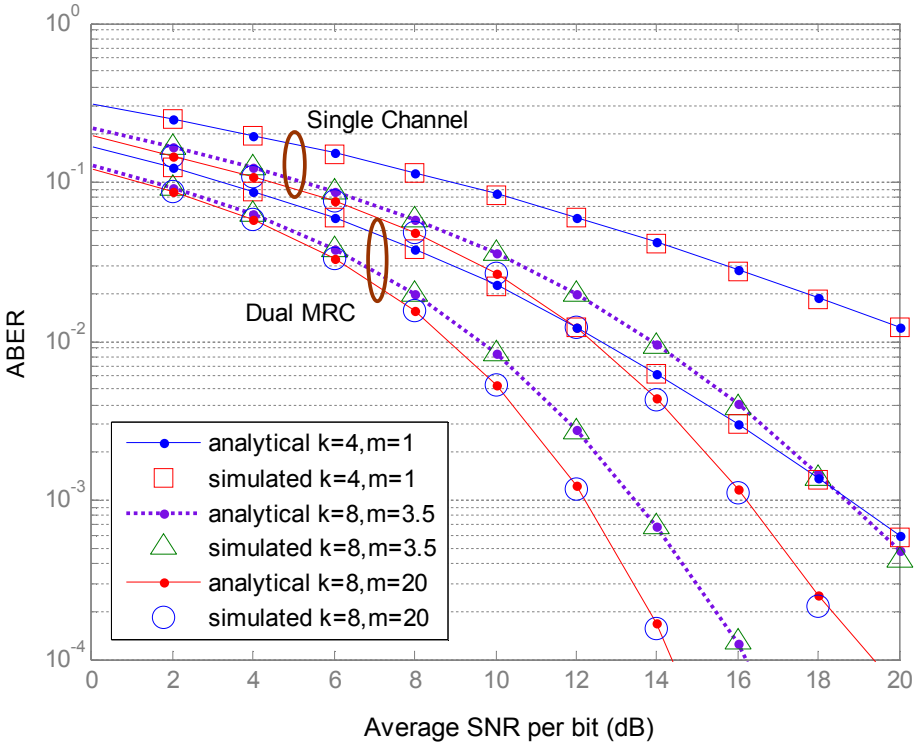

Figure 2. ABER of 16-PSK versus average SNR per bit of representative channel conditions

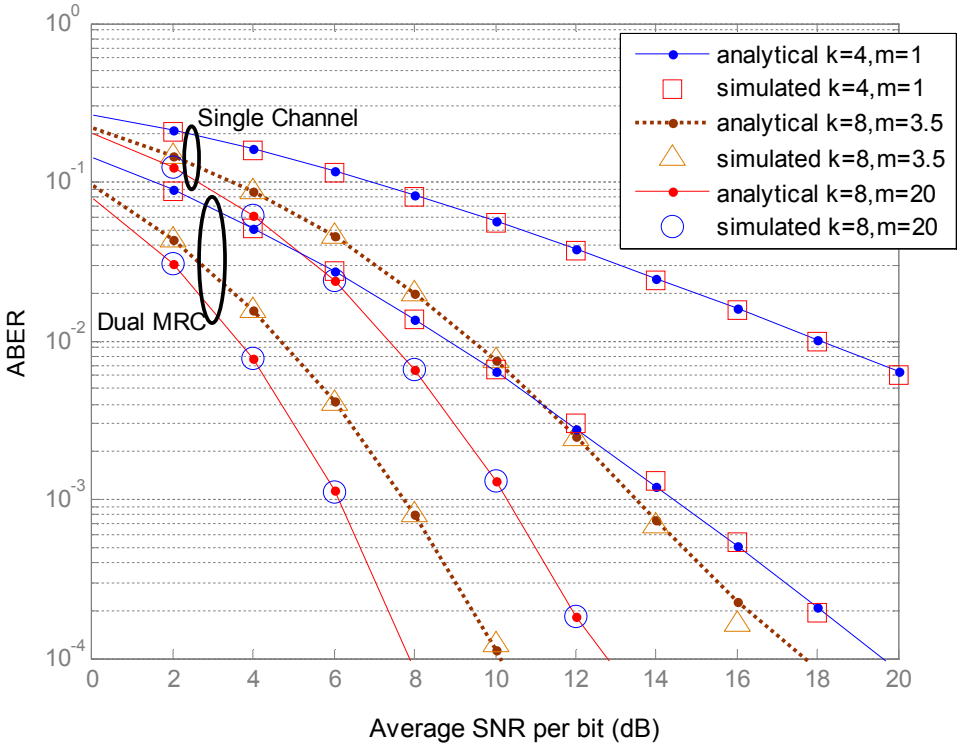

Figure 3. ABER of BDPSK versus average SNR per bit of representative channel conditions

Outage Probability

Two channels having identical average SNR and fading parameters m and k outage probabilities with and without MRC were considered and evaluated. Figure 4 shows the outage probability versus the threshold γ_{th} normalised by scaling parameter $\overline{\gamma}$. It depicts the single and dual MRC (L=2) channel receiver signal outage probability evaluated from (19) and (21) using PA and obtained via Monte-Carlo simulation. It is evident from the figure that there is a perfect agreement between both the curves. The effect of different representative channel fading-shadowing conditions through various combinations of fading parameters k and m is also illustrated in Figure 4. It is observed that as the fading parameters k and/or m increase the signal outage probability decreases. As expected, the performance of dual MRC receiver is found to be better than that of single channel receiver for any fixed value of normalised threshold. As depicted, the results obtained using PA technique and computer simulations show perfect agreement. Thus, moment-based PA method gives alternative simple-to-evaluate rational expressions, and MGF-based approach results in unified performance analysis of both single and multichannel reception employing MRC. Note that if the accuracy is not satisfactory for some cases, it is always possible to choose a higher value of D to enhance accuracy as long as the Hankel matrix is not rank-deficient. Moreover, a new set of results have been obtained here in the shadowed fading environment for higher values of fading parameters.

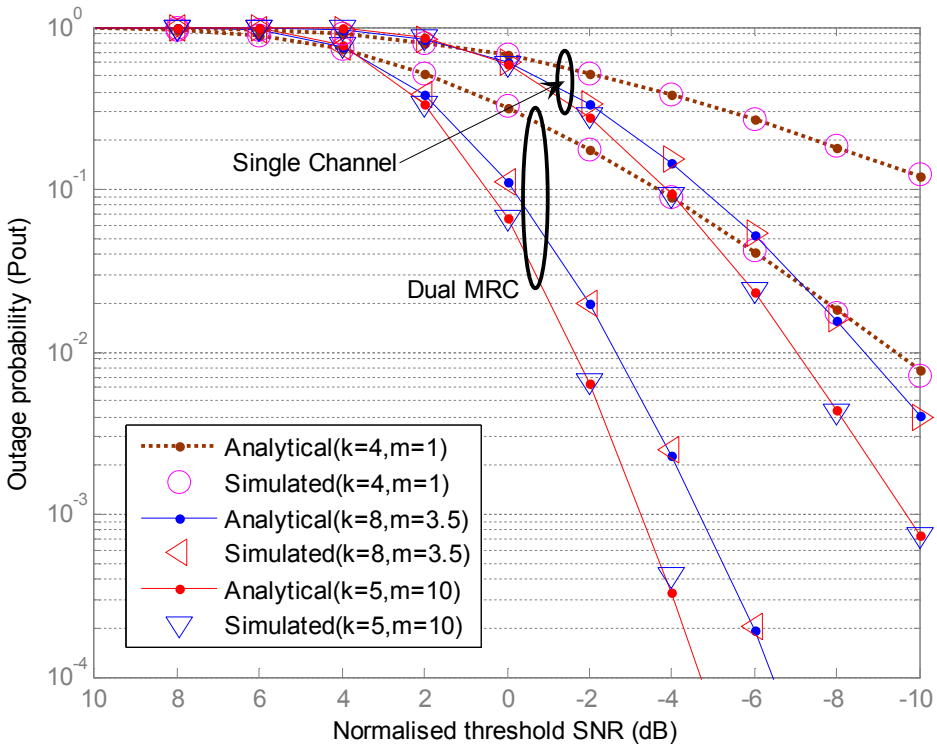

Figure 4. Outage probability versus normalised threshold in shadowed fading channel

CONCLUSIONS

The performance investigation on wireless communication systems using generic-K model was done for different shadowed-fading channel conditions. In doing so, simple-to-evaluate rational expressions for the MGF of the receiver's output SNR have been obtained using PA technique. Novel analytical expressions of ABER and outage probability for both single and multichannel receivers using MGF approach have been derived. Numerical and simulation results are presented to complement the theoretical content of the paper. It has been shown that numerical results obtained from rational expressions using PA technique and computer simulations match very well. Moreover, a new set of results for higher fading parameter values k and m have also been provided. The moment-based PA technique used here proves to be an invaluable tool for obtaining simple, easy-to-evaluate and accurate expressions for the MGF.

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Review

Biotherapy for and protection against gastrointestinal pathogenic infections via action of probiotic bacteria

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Abstract: The microbiota in the human intestine play an important function in human health and disease. Gastrointestinal infections by foodborne pathogens are a main cause of morbidity and mortality worldwide. Such infections can be caused by contaminated foods or other sources which come in contact with human intestinal epithelial cells. In recent years, probiotics have been recommended as alternative biotherapeutic agents against intestinal pathogenic infections. Two genera of probiotics, *Lactobacillus* and *Bifidobacterium*, are commercially valuable applications, several forms of which are available as capsules or in functional food products such as yogurt, fermented juices and sausages. Probiotics protect against gastrointestinal pathogenic infection via several mechanisms. These include production of antimicrobial substances, competition for nutrient substrates, competitive exclusion, enhancement of intestinal barrier function, and immunomodulation. Probiotic bacteria have been documented as being effective in biotherapeutic applications against gastrointestinal pathogens, e.g. *Helicobacter pylori*, *Salmonella*, *Escherichia coli*, *Listeria monocytogenes*, and rotaviruses. This alternative therapeutic application of probiotics to protect against gastrointestinal pathogenic infections may be of great importance for future medicinal use.

Keywords: biotherapy, foodborne pathogen, gastrointestinal infection, lactic acid bacteria, probiotic

INTRODUCTION

The human gastrointestinal tract harbours a complex and diverse ecosystem of microbiota or commensal microflora. It has been assumed that these microbiota range from 10^{12} to 10^{14} CFU/g of the luminal content [1]. There are in our body more than 2,000 different species, the majority of

which reside in the intestines [2]. Different communities of aerobic, facultative and anaerobic bacteria all constitute the gastrointestinal microbiota. The proportion of anaerobic bacteria gradually increases on going from proximal to distal areas; 99% of the inhabitants in the large intestine are anaerobes [3]. The diversity of microbiota species residing in the gastrointestinal tract is dependent upon the host's age, diet and health status [4]. Srikanth and McCormick [5] suggested that the intestinal mucosa may play a central role in host-microbiota-pathogen interactions. The human intestine is also an area which supports the energy metabolism and the immune function. Human microbiota may also play a critical role in disease and human health as suggested by Guarner and Malagelada [6] and Thirabunyanon et al [7]. Some cancers such as gastric cancer [8] and colon cancer [9] are also associated with the human microbiota and intestinal pathogenic infection. Probiotics have been promoted as new alternative biotherapeutic agents for human intestinal diseases. This report summarises the interactions between the host, microbiota and pathogens. It includes the use of probiotic bacteria as biotherapeutic agents in protection against, and treatment of, gastrointestinal infections.

FUNCTIONS OF MICROBIOTA IN THE GASTROINTESTINAL TRACT

The functions of microbiota in the human intestine consist of several main activities including metabolism, nutrition and disease protection (Table 1). Recent investigations using new techniques of molecular taxonomy have shed light on the composition, dynamics and ecology of the microbiota. Investigation of the diversity of human microbiota has revealed that this microbiota genome is at least 100 times larger than the human genome [10]. There are several types of microbial population in the human intestine such as *Lactobacillus* spp., *Bifidobacterium* spp., *Escherichia coli* and *Bacillus* spp. (Table 1). Three groups—aerobic, facultative and anaerobic bacteria—are indicated. However, the most abundant in the bacterial community are anaerobes, most of which (about 60-90%) are expressed in two divisions: the *Bacteroidetes* and the *Firmicutes* [11]. Eukaryotic fungi are also identified among the microorganisms inhabiting the intestinal tract [12].

The functions of intestinal microbiota may include diverse actions in the gastrointestinal tract including production of metabolites, nutritional fermentation and participation in the host's immune defense system. One role of human microbiota may involve maintaining nutritional homeostasis in the intestine. Nicholson and Wilson [13] suggested that several compounds produced from the microbiota co-metabolise nutrients with the host enzymes such as cytochrome P450 and conjugating enzymes in the liver. Ultimately these digested nutrients are absorbed by intestinal epithelial cells. The microbiota in the gastrointestinal tract may also produce or enrich metabolites such as glycans, amino acids, xenobiotics, vitamin K, folate and short-chain fatty acids (SCFA) [4, 10]. Starches are not easily digested by the human digestive system; however, the process is assisted by microbial fermentation. Turnbaugh et al. [14] indicated that the microbiota most able to produce SCFA are *Firmicutes* such as *Clostridium* spp. and *Bifidobacterium* spp. The primary metabolic end products of such fermentation are organic acids including SCFA such as butyrate, succinate and propionate [4, 15]. The functional roles of SCFA in colonic physiology may result in control of proliferation and differentiation of the intestinal epithelial cells [5, 16].

Table 1. Microbiota in the human gastrointestinal tract and their occurrence and/or possible functions

Microbiota	Occurrence and/or possible functions	Reference
Bacteroides spp. *Lactobacillus* spp. *Bifidobacterium* spp. *Streptococcus* spp. *Escherichia coli* *Clostridium* spp.	These bacteria originate from the birth canal and commence immediately after birth to colonise the gut; later they remain predominant in the gastrointestinal tract.	[3]
Enterococcus faecalis	Normally prevalent in healthy humans, but can cause infection under certain conditions.	[5]
Clostridium spp. *Bifidobacterium* spp.	High metabolic capacity, producing short-chain fatty acids (SCFA) within the lumen of the human gastrointestinal tract.	[14]
Pediococcus acidilactici MM33	The first human bacteriocin (pediocin)-producing strain which was found to be bactericidal against *Listeria monocytogenes*.	[34]
Lactobacillus johnsonii NCC 533	The original strain isolated from the human intestine that produces hydrogen peroxide and is effective in killing *Salmonella typhimurium*.	[36]
Lactobacillus rhamnosus IMC 501 *Lactobacillus paracasei* IMC 502	These antimicrobial strains could be used as health-promoting bacteria against harmful pathogens in humans.	[37]
Lactobacillus plantarum CS23	This strain induces potentially significant immunomodulatory activity in humans.	[38]

FUNCTION OF EPITHELIAL CELLS IN GASTROINTESTINAL TRACT

Protection of the host against intestinal pathogens is effected by the physical and chemical barriers of the gastrointestinal epithelium (Figure 1), which primarily consists of absorptive epithelial cells (enterocytes) [17]. Madara et al. [18] suggested that the human gut epithelium has a surface area of 300-400 m^2, comparable to the size of a tennis court. Epithelial cells lining the gastrointestinal tract constitute areas where contact is made between host and microbes. The structures of the apical surfaces of the epithelial cells are specialised and include microvilli, rigid intercellular junctions, and areas for ion secretion and mucus production [11]. Moreover, the microvillous tips of the epithelial cells have a surface coating of a mucous layer [17]. The intestinal epithelium also consists of several other cell types such as goblet cells, microfold (M) cells,

enteroendocrine cells, and Paneth cells. The intercellular junctional complexes are comprised of tight junctions, adherens junctions and desmosomes. The roles of these junctional complexes are to maintain the integrity of the epithelial barrier and to act as a physical barrier to prevent unwanted bacteria from entering the host [5].

The goblet cells secrete mucus in order to produce a mucous layer overlying the intestinal epithelium (Figure 1). This serves as a physical blockade protecting against harmful pathogens, which has been demonstrated with *Shigella flexneri* [19] and *Yersinia enterocolitica* [20]. The M cells differ from normal epithelial cells in that they lack microvilli on their apical surfaces. The primary roles of these M cells are in the transport of antigens, particles, macromolecules and microorganisms in the lumen through to the Peyer's patch and lymphoid tissue [21-22]. Enteroendocrine cells are hormone-secreting cells that sense the luminal environment and immediately react to secrete the correct peptide hormones such as cholecystokinin and secretin [5, 23]. The Paneth cells are another type of cells responsible for protection of the intestinal epithelium against pathogenic bacteria (Figure 1). They secrete certain antimicrobial peptides, e.g. alpha-defensins and cathelicidins. Paneth cells also produce several antimicrobial molecules including lysozyme, phospholipase A_2 and angiogenin-4 [23].

One important function of the intestinal epithelium is to create a surface where the host can sense the microbial microenvironment and generate protective responses against pathogens by producing an array of signalling molecules, e.g. chemokines and cytokines. These molecules stimulate the recruitment of leukocytes to initiate an early inflammatory response [5]. The host's immune response is expressed upon pathogenic infection; the specific recognition of molecular structures is determined by pathogen-associated molecular patterns. It has been proposed that the epithelial cells sense the microenvironment within the gut via pattern recognition receptors (PRR) including Toll-like receptors (TLRs) and nucleotide-binding oligimerisation domain (NOD) protein [24-25].

INFECTION PROCESSES OF GASTROINTESTINAL PATHOGENS

Enteric diseases are caused by several pathogens, notably *Salmonella* spp., *Escherichia coli*, *Shigella*, *Yersinia* and various other foodborne pathogenic strains such as *Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Vibrio cholerae*. *Salmonella* is known to be implicated in human foodborne illnesses and often enters the food supply via contamination of food products such as poultry, pork, beef, dairy products and nuts, especially peanut and pistachio [26]. Other strains of foodborne pathogens also typically contaminate human foods.

There are two steps in gastrointestinal pathogenic infection. At the initial stage of the infection process, the pathogens attach themselves to the surfaces of intestinal epithelial cell structures consisting of glycoproteins and glycolipids, which serve as receptors for bacterial adhesion [27-28]. *Salmonella* spp. entering via the faecal/oral route can survive in and colonise the gastrointestinal tract. Adhesion to the epithelial cells is mediated by fimbriae or pili present on the bacterial cell surface [29]. During this entry step, bacterial pathogens can pass through the epithelial barrier, triggering a proinflammatory response [30]. During the second step of the infection process, direct cytotoxic injury, intracellular migration, and disruption of the epithelial tight junctions lead to mucosal infection and systemic spread of the disease [31-32].

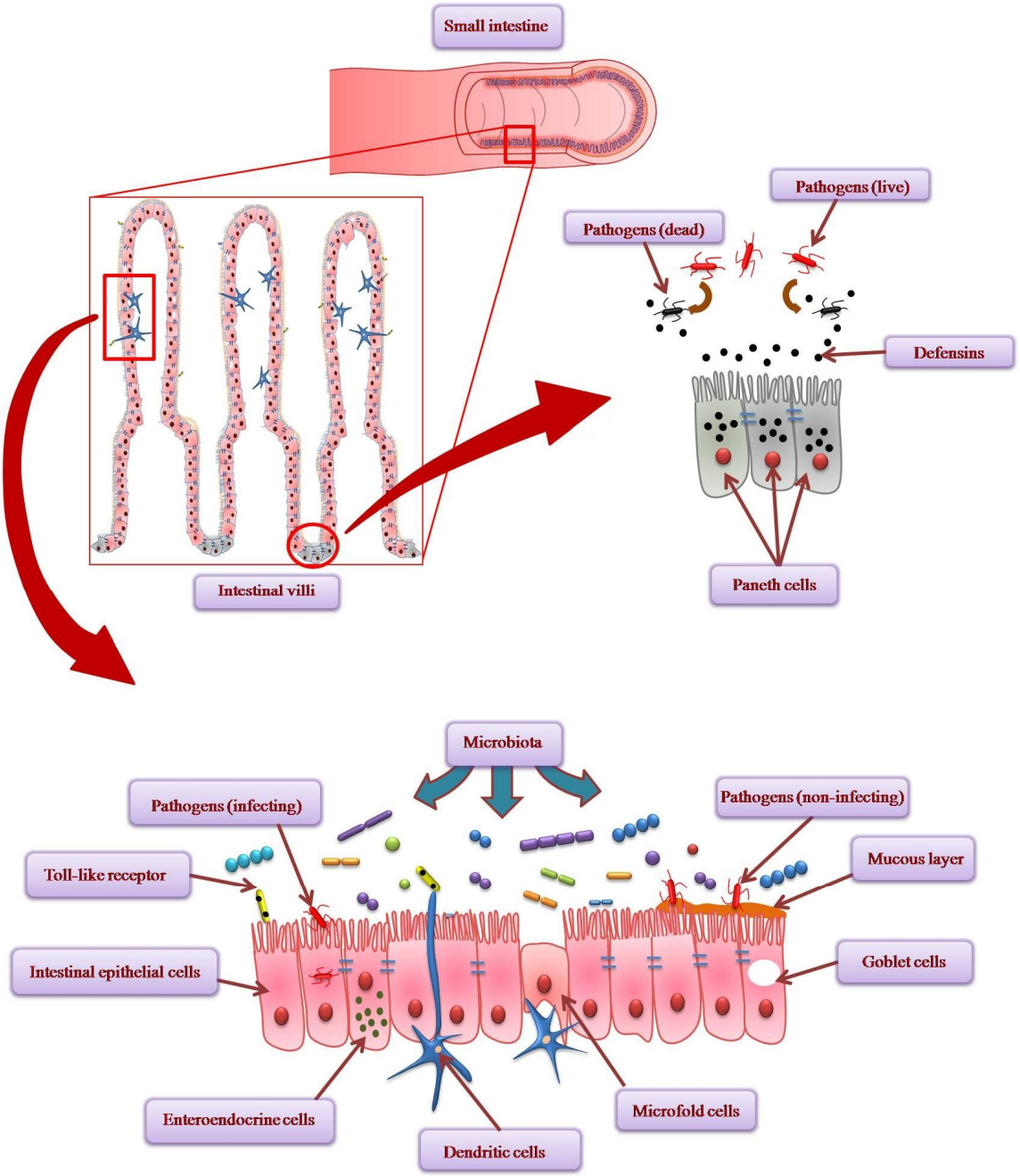

Figure 1. Functions of microbiota and epithelial cells in the lumen of human gastrointestinal tract. Intestinal microbiota are comprised of diverse groups (shown in different colours), i.e. aerobic, facultative and anaerobic bacteria, with different morphology such as rod and coccus. The intestinal epithelium consists of several cell types: intestinal epithelial, goblet, microfold, enteroendocrine, and Paneth cells. A mucous layer (brown) is a natural secretion produced by goblet cells and serves as a physical blockade protecting against pathogenic infection. Defensins (small black granules) are antimicrobial peptides secreted by Paneth cells against gastrointestinal pathogens.

THE CONCEPT OF PROBIOTICS

A probiotic is 'a live microbial food ingredient that is beneficial to health' [33]. Probiotics have recently received special attention on their application as an alternative approach to prevention of and therapy for several human gastrointestinal diseases [34-35]. Most of these potential probiotics are of human origin and are isolated from microbiota in the human gastrointestinal tract [34, 36-38]. Other sources are several human food products [39-41], which were also reported in our previous study of natural bacteria isolated from fermented milk products [7]. Recently, probiotic bacterial formulations have been developed for consumers in the forms of dietary supplements, yogurts, drinks and capsules. Two genera, *Lactobacillus* and *Bifidobacterium*, have been found to be excellent potential sources of bacterial probiotics. In addition, some species of *Enterococcus, Streptococcus* and *Bacillus* have also been suggested to have probiotic properties [7, 42-43].

Many criteria must be met to establish that a new bacteria strain is probiotic. These include non-pathogenicity, ability to inhibit the growth of pathogenic strains, tolerance for acid and bile salt conditions of the gastrointestinal tract, and ability to adhere to intestinal epithelial cells [7, 33, 44]. In vivo testing must be conducted in order to evaluate the probiotic activity in the body. If both in vitro and in vivo studies are successful, the probiotic bacteria can be used as a biotherapeutic agent in humans.

MECHANISMS OF PROBIOTIC ACTIONS AGAINST GASTROINTESTINAL PATHOGENIC INFECTION

Since the past decade probiotic biotherapeutic agents have increasingly been applied for prevention of and therapy for intestinal pathogenic infection. Consumption of probiotics may modulate the microbiota in the gastrointestinal tract and change their metabolic properties [45]. Many mechanisms have recently been postulated for these probiotic activities in the human gastrointestinal tract (Figure 2) [46-50].

Production of Antimicrobial Substances

One action of probiotics is that they can produce antimicrobial substances as direct antagonists against intestinal pathogens. Probiotics may exert their effective antagonistic activity alone or synergistically. Recent studies have indicated that the antagonistic activities against intestinal pathogens are produced by antimicrobial substances from several probiotic strains [7, 37-38]. These antimicrobial substances were found to range in size from small molecules to bioactive peptides. Bacteriocins are important ribosomally synthesised antimicrobial peptides which have been documented as possessing a good functional therapeutic activity against gastrointestinal pathogenic infection. These bacteriocins have been categorised into four classes: class-I bacteriocins are small peptides (which are also classified as lantibiotics) such as nisin; class-II bacteriocins are small, heat-stable peptides such as pediocin; class-III bacteriocins are large, heat-labile proteins such as helveticin J; and class-IV bacteriocins are complex bacteriocins [3, 51-52]. Millette et al. [34] indicated that pediocin, the bacteriocin secreted by *Pediococcus acidilactici* MM33 isolated from the human gut, was bactericidal against *Listeria monocytogenes*. Reuterin, an antimicrobial compound produced by some strains of *Lactobacillus reuteri*, may act as an antagonist against enteric pathogens [35, 53]. A study by Pridmore et al. [36] showed that the human intestinal

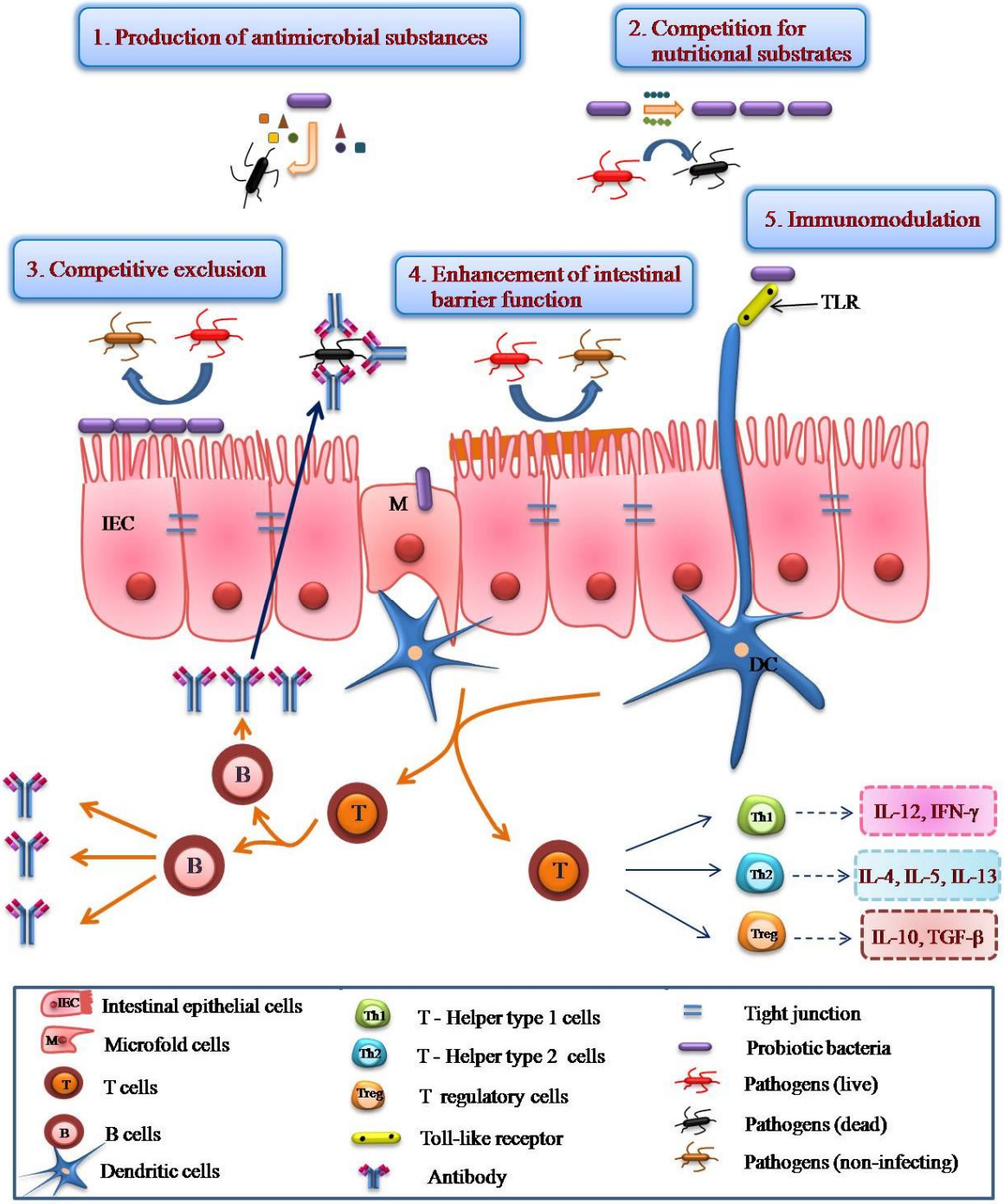

Figure 2. Schematic illustration of postulated mechanisms of probiotic bacterial actions against gastrointestinal pathogenic infection: (1) production of antimicrobial substances; (2) competition for nutritional substrates; (3) competitive exclusion; (4) enhancement of intestinal barrier function; and (5) immunomodulation.

probiotic strain of *L. johnsonii* NCC533 (La1) can produce hydrogen peroxide that is effective in killing *Salmonella typhimurium*.

Other metabolites from probiotics are potential antimicrobial substances that can protect against intestinal pathogenic infection. It has been found that five strains of *Pediococcus* spp. produce several factors that inhibit the growth of *Listeria monocytogenes*, notably hydrogen peroxide, lactic acid, exopolysaccharides, and proteolytic activity [39]. Probiotics which can produce metabolites such as acetic and lactic acids may lower the pH in the intestine. This lowering of pH results in inappropriate environmental conditions for pathogenic growth. An in vitro study by Ridwan et al. [54] showed that the antimicrobial activity of a multi-species probiotic product (Ecologic 641) may be exerted by the production of organic acids. Likewise, a biosurfactant produced from *Lactobacillus paracasei* was shown to have bactericidal activity that inhibited the growth of several pathogens [55].

Competition for Nutritional Substrates

The enteric probiotic population in the gastrointestinal tract may increase after consuming nutrients. Thus, competition for nutritional substrates amongst probiotics, intestinal pathogens and microbiota may occur. Hojo et al. [56] suggested that *Bifidobacterium adolescentis* S2-1 can better utilise vitamin K and inhibit the growth of *Porphyromonas gingivalis* by competing for the growth factor. In an animal model of germ-free mice colonised with human baby microbiota, the diverse metabolic profiles have been investigated after exposure to a probiotic strain of either *Lactobacillus paracasei* or *Lactobacillus rhamnosus*. These probiotic treatments may alter a diverse range of pathways which include the metabolism of amino acid, methylamines and SCFA [57]. Similarly, Stanton et al. [58] produced biogenic metabolites such as vitamins, fatty acids and bioactive peptides which were marked through applying probiotics in fermented functional foods. The biogenic metabolites may act as a growth substrate for selected compounds with different probiotics, intestinal pathogens or microbiota.

Competitive Exclusion

Probiotics can eliminate pathogens at the adhesion and infection site of epithelial cells in the human intestine by competitive exclusion. Infection begins with the binding of the pathogen to intestinal epithelial cells through the interaction between bacterial lectins and carbohydrate moieties of glycoconjugate receptor molecules on the intestinal epithelial cell surface [47]. Mukai et al. [59] suggested that the binding ability of *Bifidobacterium bifidum* and *Lactobacillus reuteri* to intestinal glycolipids may play an important role in their ability to adhere to the epithelial surface of the intestine. Competition study by Ramiah et al. [60] indicated that *Lactobacillus plantarum* 423 is able to colonise intestinal epithelial cells, thus preventing the adhesion of pathogenic *Clostridium sporogenes* and *Enterococcus faecalis*. These findings were similar to the author's unpublished data which indicated that a novel probiotic strain of *Bacillus subtilis* NC11 has a protective activity against *Salmonella enteritidis* infection of intestinal epithelial cells. Thus, probiotic actions against pathogenic infection can be through competitive adhesion and/or blocking of the penetration of pathogens at the infection site of intestinal epithelium cells by competing for the glycoconjugate receptors.

Enhancement of Intestinal Barrier Function

The pathophysiology of intestinal pathogenic infection displays a disruption of epithelial barrier function and a loss of tight junction formation in the intestinal epithelium cells [61]. These phenomena can increase the pathogenic or enterotoxic permeability of the mucosa wall. Probiotics have been promoted for their enhancement of intestinal barrier function by impeding the translocation and attachment of pathogenic bacteria to the intestinal epithelium [62]. Khailova et al. [63] showed in a rat model that administration of *Bifidobacterium bifidum* may have a protective effect through regulation of the main components of the mucous layer and improvement of intestinal integrity. Similarly, Mennigen et al.[64] suggested that the probiotic mixture VSL#3 can protect the epithelial barrier in a mouse model of acute colitis by maintaining tight junction protein expression and preventing the increase of apoptotic ratio.

Immunomodulation

The role of intestinal epithelial cells is associated with immunomodulation through complex interactions between immune cells and probiotics, triggering a cascade of appropriate innate or adaptive immune defense responses [47, 65]. The production of pro-inflammatory or anti-inflammatory cytokines by human peripheral blood mononuclear cells is challenged with *Lactobacillus plantarum* L2. It was found that this bacterium can induce interleukin (IL)-10 but only low levels of the pro-inflammatory cytokines TNF-alpha, IFN-gamma and IL-12. During an in vivo study, a significant increase in CD19-positive cells in the ileum was found after a daily feeding of *L. plantarum* L2 in rats [66]. Amit-Romach et al. [67] indicated that administration of the probiotic strain *Lactobacillus* GG and a mixture of *Streptococcus thermophilus*, *Lactobacillus acidophilus* and *Bifidobacterium lactis* in rats may reduce the expression of pro-inflammatory cytokines TNF-alpha and IL-6. Martínez-Cañavate et al. [68] suggested that consumption of probiotic products by children may result in enhanced innate immunity through a significant increase in natural killer cells and other specific immune factors that may improve their health status.

PROBIOTICS AS BIOTHERAPEUTIC AGENTS IN GASTROINTESTINAL PATHOGENIC INFECTIONS

Enteric pathogenic infections are a main cause of morbidity and mortality worldwide. It has been recorded that severe diarrhea and dehydration caused the deaths of 1,575,000 children under the age of five in 2006—15% of the 10.5 million deaths per year of children in this age group [69]. The enteric pathogens, notably *Helicobacter pylori*, *Salmonella enteritidis*, *S. typhimurium*, *Escherichia coli*, *Bacillus cereus, Listeria monocytogenes, Clostridium difficile*, *Campylobacter jejuni* and *Vibrio cholerae*, cause a variety of human diseases including gastroenteritis, peptic ulcer and diarrhea. These pathogens are also associated with gastric [8] and colon cancers [9]. Probiotics have been applied as alternative and biotherapeutic agents for prevention of and therapy for gastrointestinal pathogenic infections as described below.

Helicobacter pylori

Pathogenic infection by *H. pylori* can lead to chronic gastritis and peptic ulcer and increase the risk of gastric cancer [70]. *H. pylori* infection is currently treated with a proton pump inhibitor

combined with clarithromycin and amoxicillin or metronidazole [71]. Although the use of antibiotics for treatment is efficient, it is expensive and has many side effects including stimulation of antibiotic resistance in intestinal pathogens [72]. As a result, alternative application of probiotics for prevention of and therapy for *H. pylori* has been investigated. Pathogenic *H. pylori* are known to produce urease, which can hydrolyse urea to ammonium species, resulting in elevated pH in the stomach and promoting adhesion of microorganisms [73]. Thirabunyanon et al. [7] found that the potential probiotics, *Enterococcus faecium* RM11 and *Lactobacillus fermentum* RM28, isolated from fermented dairy products could inhibit the growth of pathogenic *H. pylori*. In an investigation, 14 patients infected with *H. pylori* received milk containing the probiotic *Lactobacillus casei* Shirota strain continually for 6 weeks. The results showed that urease activity declined in 64% of the patients who consumed the fermented milk, as compared with 33% for the control group [74]. Similar results were obtained by Myllyluoma et al. [75], who concluded that decreasing urease and gastrin-17 activities were found in *H. pylori*-infected patients who consumed a probiotic combination of *Lactobacillus rhamnosus* GG, *L. rhamnosus* LC705, *Propionibacterium freudenreichii* JS and *Bifidobacterium lactis* Bb12 for 8 weeks.

The suppression of *H. pylori* binding to the glycolipid receptors by the probiotic *Lactobacillus reuteri* has been reported [76]. Lin et al. [77] proposed that lactic acid bacteria isolated from commercial food products can inhibit *H. pylori* infection at the adhesion sites of human gastric epithelial AGS cells. Sgouras et al. found that *Lactobacillus casei* Shirota was highly effective in reducing *H. pylori* colonisation in the antrum and body mucosa in a mouse model [78] while *Lactobacillus gasseri* OLL2716 was shown to be effective against *H. pylori* infection in children [79]. Similarly, Wang et al. [80] indicated that regular consumption of yogurt containing *Lactobacillus acidophilus* La5 and *Bifidobacterium lactis* Bb12 may be effective in inhibiting *H. pylori* infection in humans. The outcome of using two combined probiotic strains of *Bacillus subtilis* and *Streptococcus faecium* for *H. pylori* eradication in patients were observed. These actions of the probiotic group were found to have a higher eradication rate (83.5%) than that of the control group (73.3%) [81].

Salmonella spp.

Salmonella is a major foodborne pathogen normally found in many food products. It causes many human diseases such as gastroenteritis, enteric fever, bacteremia, focal infections and enterocolitis. Human salmonellosis has become an important international public health and economic issue [82-84]. Continual use of antimicrobial agents for treatment of salmonellosis may result in the emergence of antibiotic-resistant strains of *Salmonella*. This multi-drug resistance has caused great public health concern [85-86].

The study of Thirabunyanon et al. [7] showed that lactic acid bacteria isolated from dairy products suppress the growth of *Salmonella typhimurium* and *S. enteritidis* [7]. Maragkoudakis et al. [87] observed that two food-derived probiotics, *Enterococcus faecium* PCD71 and *Lactobacillus fermentum* ACA-DC179, when co-cultured in raw chicken meat, could protect it against *Salmonella enteritidis* contamination by inhibiting its growth. A protective role of *Lactobacillus acidophilus* Bar13, *L. plantarum* Bar10, *Bifidobacterium longum* Bar33 and *B. lactis* Bar30 strains against *Salmonella typhimurium* infection of intestinal epithelial cells has been proposed [88]. Similarly, Thirabunyanon et al. found that a novel probiotic *Bacillus subtilis* NC11 strain has a protective

activity against *Salmonella enteritidis* infection of intestinal epithelial cells (unpublished data). Fayol-Messaoudi et al. [40] showed that the probiotic *Lactobacillus plantarum* ACA-DC287 strain isolated from Greek cheese can inhibit the adhesion of *Salmonella typhimurium* to intestinal epithelium cells. When mice infected with *S. typhimurium* took this probiotic, it resulted in a decrease in the levels of *Salmonella* in the intestinal tissues and contents. *Lactobacillus fermentum* ACA-DC179 was found to exert a protective effect against *S. typhimurium* infection in mice [89] while two *Lactobacillus* strains, LAP5 and LF33, showed significant antagonistic effects against *S. typhimurium* invasion of internal organs such as liver and spleen in mice that were fed the lactic acid bacteria daily for 7 consecutive days [90]. Similarly, Chiu et al. [41] found that *Pediococcus pentosaceus* MP12 and *Lactobacillus plantarum* LAP6 are able to inhibit *Salmonella* invasion in mouse liver and spleen. In another study, mice pre-fed for 7 days with milk containing *Lactobacillus casei* (probiotic dahi) prior to challenging with *Salmonella enteritidis* showed increasing production of IL-2, IL-6 and IFN-gamma, whereas IL-4 decreased in splenic lymphocytes, indicating protection against *S. enteritidis* infection by enhancement of innate and adaptive immunity [91].

Escherichia coli

Diarrheagenic *E. coli* is known to be the cause of various forms of diarrhoea and is classified into six categories, namely enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAggEC) and diffusely adherent *E. coli* (DAEC) [92-93]. Certain strains of EHEC are highly infectious pathogens that produce one or more Shiga toxins which induce gastrointestinal diseases such as diarrhoea, hemorrhagic colitis and life-threatening hemolytic uremic syndrome (HUS) in humans [94-95]. It is known that outbreaks of EHEC with serotype O157:H7 continually occur worldwide and pose a serious global health threat [96]. EHEC likely evolved from an EPEC strain; this enables EHEC to produce lesions on host intestinal epithelial cells, thus reducing intestinal epithelial barrier function [97]. EAggEC infection is associated with childhood [98] and adult diarrhoea [99] such as travellers' diarrhoea, pediatric diarrhoea and persistent diarrhoea [100]. These EAggEC strains have been observed in weaning foods, infant feeding bottles, milk and water [101]. Limited use of antibiotics for treating *E. coli* infection and alternative therapies such as application of probiotics are recommended.

Probiotic *Lactobacillus acidophilus* RY2 strain isolated from faeces of healthy infants can inhibit EAggEC adhesion to intestinal epithelial cells, thus preventing pathogenic colonisation and infection [102]. Similarly, Ostad et al. [103] concluded that the probiotic *L. acidophilus* in both live and heat-inactivated forms isolated from neonatal faeces decreases the adhesion of *E. coli* to intestinal epithelial cells. Miyazaki et al. [93] demonstrated that a probiotic strain of *Enterococcus faecium* has bactericidal effects on EAggEC by inducing membrane damage and cell lysis. Protection of the tight junction of intestinal epithelial cells against EHEC-induced damage has been found via the activity of probiotic *Bifidobacterium lactis* 420 strain [104]. Mangell et al. [105] pre-fed rats with probiotic *Lactobacillus plantarum* 299v strain in drinking water and then challenged them with an *E. coli*-induced increase in intestinal permeability. The results showed that this probiotic strain can exert a protective effect. A comparison of probiotic feeding with *Bifidobacterium thermacidophilum* RBL71 for 7 days before and after infection with EHEC *E. coli* O157:H7 in mice was investigated.

The effects were greater in the pre-challenged group compared to the after-treatment group, resulting in increased feed intake and weight gain and lower faecal levels of *E. coli* O157:H7 [106].

Listeria monocytogenes

L. monocytogenes has been found to be a contaminant in various raw and processed foods such as beef, pork, sausages, milk, dairy products, vegetables and seafood products [107-108]. It causes listeriosis, a foodborne pathogenic illness that primarily infects pregnant women, newborns and elderly or weakened individuals [109]. *Listeria* has also been implicated as the cause of septicemia, spontaneous abortion and even death of infected individuals [110]. The mortality rate of this illness may reach 20-30%, making it a serious public health menace [107]. *L. monocytogenes* is known to tolerate environmental stresses including variations in pH, temperature and osmolarity [111]. Because it can survive in foods for long periods of time, it has been implicated in outbreaks in meat and dairy products [112-113].

Infection by *L. monocytogenes* may translocate from the gastrointestinal tract to other organs such as liver, spleen, central nervous system and placenta [114]. Several biotherapeutic agents for *L. monocytogenes* infection have been investigated. De Waard et al. [115] demonstrated that rats fed *Lactobacillus casei* Shirota YIT9029 strain continuously for 3 days before being infected with *L. monocytogenes* show reduced levels of the pathogen in the faeces and several organs, i.e. stomach, caecum, spleen and liver. Corr et al. [116] observed the anti-infective activity of *Lactobacillus salivarius* UCC118, a strain of human origin that produces Abp118 bacteriocin which can protect against *L. monocytogenes* infection in mice. In another study, after orally feeding *Lactobacillus plantarum* to mice continuously for 30 days and then challenging by intravenous infection with a clinical strain of *L. monocytogenes*, it was found that the administration of *L. plantarum* reduces pro-inflammatory interleukin (IL-1 beta and IL-6) production, implicating the host protection against *L. monocytogenes* [117]. Similar results were found in mice treated with *Lactobacillus delbrueckii* var. *bulgaricus* UFV-H2b20 and challenged with *L. monocytogenes*. The mice were more resistant to this pathogenic infection, as registered by mortality rates and number of bacteria in spleen and liver. They also showed increasing production of inflammatory cytokines (interferon-gamma and tumor necrosis factor-alpha) and nitric oxide [118].

Clostridium difficile and rotavirus

Evaluation of potential probiotics for their ability to protect against infection by other intestinal pathogens has also been undertaken. Effective probiotic treatments of *C. difficile* infection which causes gastrointestinal illness have been proposed [119-122]. Protection against rotavirus infection which is a leading cause of gastroenteritis, especially in young children, has also been investigated [123-124].

CONCLUSIONS

Recently, human diseases and probiotic bacteria have become interrelated fields of investigation through the association with gastrointestinal infections from foodborne pathogens that are known to be a main cause of morbidity and mortality worldwide. Hence, many studies are now in progress on the applicability of probiotic bacteria as an alternative biotherapeutic treatment for, and

protection against, gastrointestinal pathogenic infections. Probiotic bacteria are derived from human microbiota; since they are of human origin, they may have key features as primary sources for human disease therapies. New sources which originate from fermented foods are also significant for both functional food development and alternative biotherapies. One important limitation is that only one kind of probiotic bacteria may not exert protection against all harmful strains that cause gastrointestinal pathogenic infections. Therefore, effective investigations of individual strains of probiotic bacteria and also of new formulations that combine several probiotic activities in challenging certain gastrointestinal pathogens—in vitro, by cell culture, and in animal models as well as in humans as a final evaluation—are necessary before a biotherapeutic application. Biotherapy with probiotic bacteria for gastrointestinal pathogenic infections may modulate functions of the microbiota and intestinal epithelium in the gastrointestinal tract, resulting in many documented action modes such as antimicrobial production, nutritional substrate competition, competitive exclusion, intestinal epithelial function, and immunomodulation. The present investigations of this alternative biotherapeutic application of probiotics to protection against gastrointestinal pathogenic infections may be of great importance for both present and future medicinal use.

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Report

Nature conservation in urban conditions: A case study from Belgrade, Serbia

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Abstract: This paper analyses the Serbian nature protection system in Belgrade. Effective protection of natural features in urban landscapes have become increasingly complex due to conversion of natural habitats, high levels of pollution and other forms of deterioration caused by human impact. These anthropogenic pressures vary in type and intensity and depend on the location of protected assets. Through comparative analyses of selected legally protected natural assets in various areas in the city, different features of spatially-functional coexistence and development are noted. According to the results of this research it is evident that various natural protected assets in highly urbanised conditions can sustain theirs primary ecological function and can also develop additional adjusted functions during time.

Keywords: nature conservation, nature protected assets, Belgrade, urban landscape, human impact

INTRODUCTION

The modification of landscape structures and functions by human development is an immutable fact of contemporary society. The main differences between urban and non-urban ecosystems are the type, intensity and frequency of anthropogenic influence. Urban ecosystems are governed by human actions and it is important to consider these actions when studying urban ecology [1]. The spatial disposition, survival and functions of autochthonous ecosystems depend increasingly more on anthropogenic interventions. Their protection is often undertaken too late to prevent the destruction or deterioration of natural assets. Ecosystems have the greatest degree of sensibility to human influences, but this also applies to abiotic natural resources (geological,

hydrological, etc.) that are situated in urban landscapes. Urban landscapes are a complex mosaic of autochthonous covers (geological, pedological, hydrological, vegetation, etc.) altered by anthropogenic actions and land use.

Typically, cities show a mosaic of habitats with increasing degrees of human impact along a gradient from the outskirts to the city centres [2]. Similarly, Bryant [3] states that "it is clear that habitat diversity and life-support conditions vary greatly along the urban-rural gradient. For conservation purposes, it is important to consider the full spectrum of environmental conditions, from urban core to urban fringe, when planning interventions." Urban land uses are in a state of continuous flux in which change is the norm rather than the exception [4]. Also, from particular interest in research as presented in this paper are landscapes situated on the city edge. As Qviström and Saltzman [5] pointed out for Malmö, Sweden, "a critical analysis of landscape dynamics at the inner fringe can highlight the ways in which time and space are understood within landscape studies."

The main factor determining living conditions in cities is human impact. Increased human densification means that the maintenance and conservation of tracts of natural or semi-natural vegetation will become more difficult in areas of higher human density [6]. With increased human densification, densely built-up areas also expand, causing the loss of biotope structures and the introduction of different species. The effects of urbanisation are more intense in the inner city and this can support specialised urban plant communities [7]. Massive disturbances created by city growth not only destroy the habitats of native species, but also create habitats for the relatively few species that are able to grow in urban and suburban conditions [8], which may lead to urban biotic homogenisation. According to Maurer et al. [9], "for a long time nature conservation in cities has been restricted to relicts of the natural landscape such as wetlands or woods. However, for a more general evaluation of urban land-use types, nature conservation in cities should focus not only on primary natural assets, but also include other important and more or less subjective arguments. All urban land-use types reflect the historic artificial landscape as well as the economic and cultural changes in cities. The vegetation thus represents relicts of historical and botanical heritage."

During the collections of data for our research, one of the problems that appeared was an inconsistency in the defining terminology and in the essential recognition of different features and areas of this type. Löfvenhaft et al. [10] state that the basic problem in the collection of such data occurs due to "poor and/or heterogeneous compilations of ecological data and the terminological confusion leading to multiple meanings for terms such as nature, forest, park, built-up area and green area." For Belgrade, which is the subject of this study, it is obvious that relevant legal documents clearly define the concepts of green areas, forest parks, forests, parks and protected natural assets. However, in official documents and among the public, confusion in the use and essential properties of such terms is present. This clearly results in a remarkably complicated or impossible comparison in an international context, especially in countries where legislation is not compatible with EU regulations and recommendations, as is the case with Serbia.

We aim to present an analytical survey of natural protection in Belgrade, especially from the perspective of spatially-functional coexistence within this environment. For this reason, a comparison of different protected natural assets based on their qualifications to legal protection regimes and locations in the city will be examined, along with the possibility of their sustainable ecological

existence. For the purpose of comparison within the same legal type, the selection is based on pairs of protected assets which are creation limited mostly by natural versus anthropogenic influences. Our research will attempt to determine whether and to what extent the location of protected assets in a gradient from the city edge to city centre affects their functions.

GENERAL OVERVIEW OF STUDY AREA

The city of Belgrade, capital of Serbia, covers an area of 322,268 ha, which is 3.65% of the total area of the country. The coordinates for Belgrade are: 45°06' N, 44°16' S, 44°27' E and 44°38' W. The highest elevation is 628 m, measured at a distance of 50 km south-east of the narrow city core. The lowest elevation at the most downstream portion of the Danube River in city is 71 m.

Belgrade is situated in south-east Europe at the confluence of the Sava and Danube Rivers, at the boundary between the Pannonian plain and Balkan Peninsula. In Belgrade's territory, Neogene deposits are far more prevalent than Mesozoic formations [11]. Its geographic location and relief characteristics give it a mild-continental climate with an average annual temperature of 12.7°C and annual precipitation of 750 mm.

Due to such geographic conditions, the autochthonous vegetation consists of a mosaic of grass formations in the northern part of the city on the pedological substratum of hydrogenic and chernozem soils, and forest vegetation in which, in a zone from Sava and the Danube River towards the south, associations with dominant species, namely *Salix alba* L. (Salicaceae) and *Populus alba* L. (Salicaceae), alternate with a thermo-mesophilous oak forest zone of the West-Moesian sub-region. The forest associations that were widely distributed in the past with most widespread species such as *Quercus cerris* L. (Fagaceae), *Quercus frainetto* L. (Fagaceae) and *Carpinus betulus* L. (Corylaceae) are now usually only present in various degraded forms [12-13].

Because Belgrade's population history dates in continuity from the 1st century B.C., the autochthonous vegetation cover has been greatly altered by anthropogenic influences, which is of great importance for research on this issue. Today, the city's territory is divided into 17 municipalities, where 1,689,000 inhabitants or 22.5% of the total Serbian population live.

Nature Conservation by Law in Serbia

The nature conservation system in Serbia was created on a legislative basis according to the Law on Nature Protection [14]. The basic unit of protection is known as a protected natural asset, which is defined as a 'preserved area of nature, with particularly great natural values and features which give to the area a permanent ecological, scientific, educational, recreational or other importance. So, being of particular interest, this asset is particularly protected.'

In addition to the general definition, the Law on Nature Protection provides seven different types of protected natural assets, viz. national park, nature park, landscape of outstanding features, natural reserve, special nature reserve, natural monument and natural rarities. Brief descriptions of these types of protected natural assets are shown in Table 1.

Table 1. Definitions of protected natural assets in order of importance

Protected natural asset	Definition
National park	Large area with highly valuable, well-preserved natural ecosystems, with complex structural or biogeographical features with diverse original flora and fauna, representative physical-geographical phenomena and with cultural-historical value such as an exceptional natural unit important for the country.
Nature park	Area of well-preserved natural water, air and soil, dominant natural ecosystems, and without major degradation changes of landscape; a significant area of preserved nature and healthy environment.
Landscape of outstanding features	Relatively smaller area with vivid landscape features, undisturbed primary values of landscape with a presence of traditional ways of life and cultural heritage; also the protected surroundings of immobile cultural assets.
Natural reserve	An original or minimally altered area of nature of a special composition and features of plant or animal communities within the ecosystem; intended primarily for the preservation of genetic funds.
Special nature reserve	Area with one or more natural values to be particularly protected or natural phenomena to be observed and managed.
Natural monument	Natural object or phenomenon, physically clearly expressed and recognisable with representative geomorphologic, botanical or other features (geological, hydrological, etc.), which, as a rule, ought to be of attractive and remarkable appearance or mode of appearing; also a man-made artificial botanical value (individual trees, arboretum, botanical gardens, etc.) if it has particular importance.
Natural rarities	Plant and animal species or communities whose survival is threatened in their natural habitats or whose populations are quickly becoming extinct or whose habitats are degraded, or species that have narrow distributions or have special ecological, genetic, economical, sanitary or other significance.

Different levels of natural asset values dictate implementation of various management methods and responsibilities for the care, protection and improvement of some natural assets. Accordingly, many requirements are needed for categorisation of protected natural assets in order to improve management of protected areas. Under the Serbian Law on Nature Protection, it is possible to recognise the following categories of protection, i.e. category I: exceptionally important natural assets; category II: very important natural assets; and category III: important natural assets.

The procedure of categorisation and evaluation of protected natural assets is under the jurisdiction of the Institute for Nature Conservation of Serbia, which prepares expert reviews and conducts evaluations and proposes a category for each protected natural asset. The categorisation

procedure is based on the natural assets' essential features such as authenticity, being representative, relict or endemic, rarity, degree of preservation, functions and importance of assets (ecological, scientific, educational) as well as the level of endangerment to the protected natural assets.

CASE STUDY

Approximately 550,000 ha or 6.19% of the total area of the Republic of Serbia is protected by the Law on Nature Protection. The area covered by this research consists of approximately 5,000 ha of protected areas, which is about 1% of the total protected natural assets in Serbia.

In the research area, the tradition of legitimate nature protection is 40 years old. It was intensified in the last decade (see Table 2), which corresponded to the period of greatest anthropogenic pressure (spatial expansion of the urban zone and a population increase of approximately 100,000 from 1991). Mostly, the data were collected from the national institution for nature protection, Institute for Nature Conservation of Serbia, and were supplemented with empirical data. Table 2 provides a list of legitimate protected natural assets in Belgrade, but it does not include a detailed list of individual protected trees.

According to the Serbian Law of Nature Protection, among natural monuments it is also possible to find individual trees with protected status. In Belgrade, 35 natural monuments of botanical character are registered as protected trees, among which some exotic and decorative species are dominant that do not belong to the autochthonous floristic elements (e.g. *Cupressus arizonica* L. (Cupressaceae), *Liriodendron tulipifera* L. (Magnoliaceae) and *Platanus acerifolia* L. (Platanaceae)—the oldest tree in Belgrade planted in 1839). From the category of autochthonous floristic elements, only a few trees are protected (only nine in the four localities and all are the same species, namely *Quercus robur* L. (Fagaceae). This is evidence of a significant deterioration of the vegetation cover of Belgrade, which has been almost fully destroyed by urban development. Although protected trees will not be a subject of detailed discussion in this paper, their imposing visual prominence in the ambient environmental picture, in addition to explanations of their scientific importance and their need for protection, has contributed to the popularisation of the idea and practice of nature protection even in a highly developed urban landscape such as that of Belgrade.

Among the protected natural assets shown in Table 2 are landscapes of outstanding features and natural monuments with different protection values. Forests and park complexes are dominant, but among them are also three natural monuments of geological importance. Protected assets such as natural monuments of geological significance have exclusive scientific, educational and cultural/historical value and, because of their particularity and on the basis of their area and location, do not have limiting effects on the development of surrounding areas.

We will emphasise two landscapes of outstanding features—Veliko ratno ostrvo and Avala—and two natural monuments—Banjička šuma and Jevremovac Botanical Garden (Figure 1) to elucidate the spatially-functional coexistence of nature conservation and urban development.

Table 2. Protected natural assets in Belgrade

No.	Protected asset	Area (ha)	Asset type	Year of protection	Main value
1.	Avala	489.13	LOF	2007	Well-preserved mountainous thermo-mesophilous oak forest habitat
2.	Kosmaj	3514.50	LOF	2005	Well-preserved mountainous thermo-mesophilous oak forest habitat
3.	Veliko ratno ostrvo	167.9056	LOF	2005	Ecosystem of typical wetland vegetation and avifauna
4.	Banjička šuma	58.6586	NM	1993	Ornithological site
5.	Miljakovačka šuma	244	NM	2008	Preserved thermo-mesophilous oak forest habitat
6.	Topčider	12.83	NM	2008	Public park site with many protected trees
7.	Košutnjak	267	NM	2008	Preserved thermo-mesophilous oak forest habitat
8.	'Jevremovac' Botanical Garden	4.8183	NM	1995	University botanical garden
9.	Akademski Park	1.4590	NM	2007	Public park site with many protected trees
10.	Pionirski Park	3.6013	NM	2007	Public park site with many protected trees
11.	'Mašin majdan' Senonian shelf	4.5	NM	1969	Rare geological site
12.	Sea Neogene shelf – Kalemegdan	0.006	NM	1969	Rare geological site
13.	Miocene shelf – Tašmajdan	2.5	NM	1968	Rare geological site
14-48.	Protected trees	1.5	NM	1969-	Remarkable trees

Note: LOF = landscape of outstanding features; NM = natural monument
Source: Institute for Nature Conservation of Serbia [15]

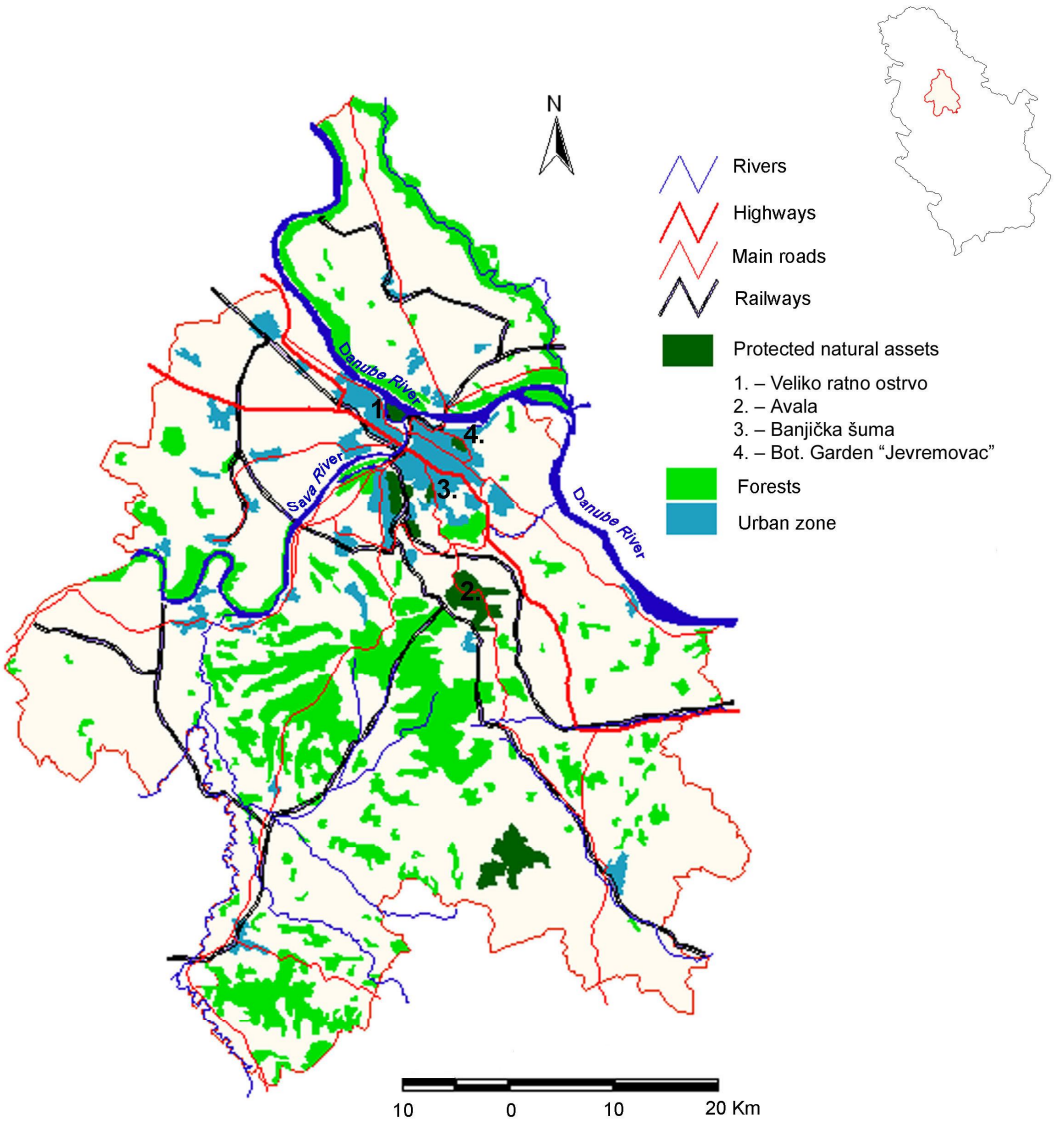

Figure 1. Distribution map of selected protected natural assets in Belgrade area

The Veliko ratno ostrvo (Figure 2) is an area of outstanding features, representing one of the largest areas and the most composite landscape complex with its physical-geographic, biotic and ecological particularities that is protected in Belgrade. It is located in the narrow city centre and it lacks human occupancy. This island originated from the accumulation of fluvial deposits at the mouth of the Sava River next to the Danube. The habitat is of seasonally flooded forest with *Salix spp.* and *Populus spp.* and wetland vegetation. The vegetation supports a rich avifauna on the island with nesting and feeding habitats. The number of birds vary depending on the water level and the season, but the most common species among 160 species seen on this island are: *Egretta garzeta* L. (Ardeidae), *Larus ridibundus* L. (Laridae), *Podiceps cristatus* L. (Podicipedidae), *Ciconia ciconia* L. (Ciconiidae), *Phasianus colchicus* L. (Phasianidae), *Circus aeruginosus* L. (Accipitridae), *Accipiter gentiles* L. (Accipitridae) and *Buteo buteo* L. (Accipitridae) [16].

Figure 2. Veliko ratno ostrvo at the mouth of Sava River (left) on the Danube (right), 2010

Avala, a landscape of outstanding features that includes a low mountain (506 m) of the same name (Figure 3), is about 15 km away from the city centre. The dominant ecosystem of Avala consists of a mountainous thermo-mesophilous oak forest habitat of the West-Moesian floristic subregion [16]. Almost 70% of the total area is covered by forest within the altitude region of oak and beech habitats with most common species such as *Quercus frainneto* L. (Fagaceae), *Quercus cerris* L. (Fagaceae) and *Fagus moesiaca* (K.Maly) Czeczott (Fagaceae) in association with *Ruscus aculeatus* L. (Gonvallariaceae). Among others, dominant species such as *Tilia tomentosa* Moench. (Tiliaceae) and *Fagus moesiaca* are also found on the Avala mountain. Anthropogenic interventions to increase the forested area and to artificially enrich flora were done more than 100 years ago, with introduced trees such as *Pinus nigra* Arn. (Pinaceae), *Pinus silvestris* L. (Pinaceae) and *Cedrus atlantica* (Endl.) Carr. (Pinaceae) being planted. In this oak and beech habitats, numerous bird species have been found, most notably *Falco tinnunculus*, *Strix aluco*, *Otus scops*, *Buteo buteo* and *Dendrocopos major*. Some species of flora and fauna on Avala mountain, e.g. *Thlaspi avalanum* Pancic (Brassicaceae) and *Strix aluco* L., are protected by Serbian law of natural rarities [17].

Figure 3. Panoramic view of Avala mountain, 2010

Banjička šuma woodland (Figure 4) is protected as a natural ornithological area and is located approximately 5 km south of the city centre. The forest is dominated with anthropogenic trees, viz. *Quercus robur* L. (Fagaceae), *Acer campestre* L. (Aceraceae), *Populus virginiana* Foug. (Salicaceae) and *Cedrus atlantica* (Endl.) Carr. (Pinaceae), planted in the primary habitat of *Tilia tomentosa* Moench. (Tiliaceae) and *Quercus spp.* during 1948-1950. It has developed into three levels that serve as habitats for 68 bird species, of which 40 are residents, 16 are migrators and 12 are migrants. Four of these species (*Oriolus oriolus, Turdus pilaris*, *Strix aluco* and *Buteo buteo*) are also strictly protected by the Serbian regulation for natural rarities [16].

Jevremovac Botanical Garden is situated in the city centre, where vegetation conditions are unfavourable, i.e. there is high air pollution. This natural monument is a habitat for more than 1,000 outside plant species and an additional 1,000 species in the indoor part of the garden. Since the artificial ecosystems of the botanical garden has specific conditions, it is also a habitat for 25 of the 58 moss species in Belgrade [18].

Figure 4. Banjička šuma, 2009

Figure 5. Jevremovac Botanical Garden, 2009

Methods

The first step entailed a detailed survey of the literature relevant to this research. This included the flora-vegetation, avifauna and ecosystems. By study of contemporary urban ecology papers, it was concluded that flora-vegetation criteria are crucial in decisions for the enactment of nature protection laws [9, 19-26]. Avifauna diversity and richness were of special importance for protection in the literature [27-31]. The theoretical basis of analysis also included relevant literature [4, 32-41]. We found a few suitable inventories of autochthonous plants and fauna in Belgrade [12-13, 16].

We then did a qualitative comparative analysis. The following criteria were used: qualification to the same type of protected assets on a legal basis, spatial location of assets (centre vs. outskirts), and genesis (slightly altered natural ecosystem vs. initially dominant ecosystems of anthropogenic origin).

The final phase of comparative analysis involved a critical overview of the selected examples with special focus on their spatially-functional coexistence. With that aim in a mind, data about biodiversity and ecosystems as well as disturbances were reviewed.

RESULTS AND DISCUSSION

The result of a comparative analysis performed on two pairs of protected areas that belong to the same category of protected assets is presented in Table 3. The first pair consists of two natural assets that are classified as landscapes of outstanding features, Veliko ratno ostrvo and Avala, and the second pair as protected natural monuments, Banjička šuma and Jevremovac Botanical Garden.

Table 3. Comparison of selected natural assets

Criteria		First pair		Second pair	
		Veliko ratno ostrvo	Avala	Banjička šuma	Jevremovac Bot. Garden
Type of protected natural assets	Landscape of outstanding features	+	+	-	-
	Natural monument	-	-	+	+
Location	Centre	+	-	-	+
	Outskirt	-	+	+	-
Genesis	Natural ecosystem	+	+	-	-
	Disturbed ecosystem	-	-	+	+

During the comparison of the first pair of natural protected assets we found that both have remarkable and unique ecological values (Table 4). The location of these natural protected assets does not affect their ecological and scientific functions. It was also found that location does not influence the existence of limited production activities (e.g. agriculture and forestry) which are allowed by the Serbian law on nature protection.

Table 4. Functions of selected natural assets

Function	Veliko ratno ostrvo	Avala	Jevremovac Bot. Garden	Banjička šuma
Ecological	+	+	+	+
Scientific	+	+	+	+
Educational	-	-	+	-
Recreational	+	+	-	+
Productional	+	+	-	-
Residential	-	+	-	-

Educational function only recorded for one of these protected assets due to absence of guide service and not to their location. The location of these assets does not influence their recreational function. For Veliko ratno ostrvo the recreation function is limited only to the summer because it is an inaccessible and inhospitable island during the rest of the year (flooded areas, lack of the trails, etc.). A residential function is therefore not applicable here while Avala has this function (mostly rural households) because of its large area. Finally, we can say that the presence of all these functions in the protected assets analysed is not influenced by their location.

These examples belong to the same type of protected natural assets not because of compatible ecosystems or landscape values (wetlands or thermo-mesophilous mountain forest ecosystem), but rather because of the similar magnitudes of urban development suffered by both places. The types of pressure and the forms of autochthonous values at risk are different though. At Veliko ratno ostrvo, the endangering factors are continuous and affect all habitat types directly and equally. Conversely, for Avala the endangering factors are sporadic and directly devastate only forest habitats. We determined that significant functional differences in the analysed examples do not derive from their different spatial connections with the urban core.

A comparison of the second pair shows that both protected natural assets have specific ecological and scientific functions. In the case of Jevremovac Botanical Garden, where allochthonous conditions are suitable for the largest number of plants, the ecological, scientific and educational functions are high. A cultural-historical dimension is also present with Jevremovac Botanical Garden, which was created more than 120 years ago. Banjička šuma also has ecological and scientific functions but lacks educational values as a result of a deficiency in management activities. It is obvious that location does not have any influence on the functions of the second pair of protected assets. Neither of these has productional or residential function. Recreation (walking, tracking, jogging or bicycle riding) is not a common characteristic of Jevremovac Botanical Garden while Banjička šuma is a popular area for the same activities in the local surrounding.

The natural assets of Banjička šuma and Jevremovac Botanical Garden were included in this research because of similarities in their initial phases of genesis since both were planned for a specific purpose relatively recently and have achieved various functions. The primary function of Banjička šuma was the afforestation of devastated land. With time it developed spontaneously from direct management-oriented activities into an optimal habitat for birds. Jevremovac Botanical Garden was operational for more than a century and has become ecological filter in the most urbanised and

polluted part of Belgrade. In accordance with legal conservation postulates, it has stimulated the protection and improvement of not only the original, autochthonous ecological functions of those sites, but also of those that have developed in dynamic interactions between the abiotic features of the urban environment and the biotic potentials of the same areas.

According to the presented results, it is clear that ecological and scientific functions of all selected protected assets are well exposed and not influenced by location (Figure 6). Such results are expected since improvement of these functions is the primary reason for their legal protection.

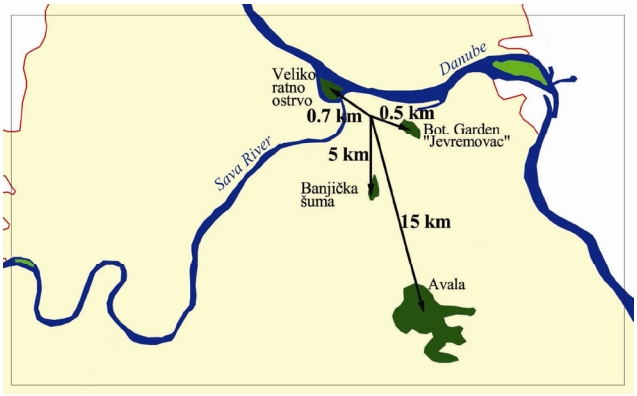

Figure 6. Detail of the study areas with distances from the city centre

Considering this in the time scale, it was observed that for landscapes of outstanding features (Veliko ratno ostrvo and Avala), definite conclusions about changes in their ecological and scientific functions are too early to know. It is evident that some disturbing factors have been minimised. Research on Veliko ratno ostrvo by Šinžar-Sekulić et al. [36] noted that "twenty years ago, 53.2% of the island were agricultural areas, followed by 28.4% of willow and poplar forest. Nowadays, only 6.0% of the island is used for agriculture." By undertaking adequate protection measures, it has been possible to reduce the exploitation of autochthonous habitats and landscapes. Anthropogenic influences are causes of environmental disturbance (intensive fluctuations of ground water level, pollution of surface and ground waters, decrease of area under indigenous vegetation, extermination of avifauna habitats, disturbance of habitat conditions for ichthyo- and herpetofauna, etc.). The specific location of Veliko ratno ostrvo wetlands is at a disadvantage for the regeneration of its ecosystem. Because of its isolation and lack of contact with related ecosystems, the nearest being at least 10 km away, measures to manage this natural asset have been oriented towards the stimulation of the sustainable capacity of the ecosystem. At Avala, minimising clear cutting of forest cover and degradation of biodiversity has been the main reason for the initialisation of protection. Breuste [22], states: "To preserve indigenous vegetation, it is necessary to understand the forces on the processes of urban growth as well as the ongoing landscape changes by agriculture and forestry, and how to deal with this transformation of landscape." This is pursuant to the findings of Zhu and Zhang [25], who emphasised that "in a dynamic context, we see that the eco-regional condition may influence the changing amount of urban forest land during different stages of city growth."

For the natural monuments, i.e. Banjička šuma and Jevremovac Botanical Garden, where legal protection has been in force for longer than one decade, it is possible to follow improvement of their ecological and scientific values. At the Jevremovac Botanical Garden, according to Jovanović [42], "among other initiatives, a program of ex situ protection of endangered Serbian plant species is being developed with the goal of reintroducing those species to the natural habitats from which they have disappeared." This place also operates as an ecological filter: typical unpolluted air bio-indicators (related to limited values of SO_2 concentration) have been found according to Cvijan et al. [39], who also reported six species of lichens in the Jevremovac Botanical Garden, which represents an isolated island within the 11-km^2 "lichen desert" of Belgrade. As can be seen in Figure 1, the location and elongated shape of Banjička šuma functions as an ecological corridor for the avifauna of southern Belgrade. Such corridors stabilise the survival of mobile habitat-dependent species [27]. These should not be substitutes for the protection of large, intact nature reserves in urban or suburban landscapes [28]. Banjička šuma is situated at the intermediate point along urbanisation gradient and has significant richness of avifauna [35]. According to well-known landscape ecology postulates, small natural vegetation patches serve as stepping stones for species dispersal and provide heterogeneity. In urban conditions, it is more difficult to successfully provide the level of protection available to undisturbed regions of larger areas. We agree with Forman [43] that "small patches provide different benefits than large patches and should be thought of as a supplement to, but not a replacement for, large patches." Diversity of habitat patches is a result of stochastic colonisation events combined with varying degrees of human-induced disturbance [44]. According to Colding [4], it is believed that "ecological land-use complementation may involve the clustering together of a whole range of different green patches in cities to increase available habitat and promote ecological processes." Although Banjička šuma is surrounded by dense urban development and very heavy traffic, the healthy state of this ecosystem indicates that it has resisted negative anthropogenic influences. Whatever the biodiversity quality of the periurban landscape, site-specific actions such as shrub and tree planting, water table restoration and increasing vegetation diversity can change bird diversity and improve the quality of Nature [29].

For a better understanding of the avifauna of Veliko ratno ostrvo and Banjička šuma, it is necessary to undertake future inventory research by using "a habitat island approach which may be a good starting point for bird management in urban landscapes" [30]. Future research on the effects of human disturbance on urban birds should be directed to the analysis of the relationships between human disturbance and tolerance levels for more skittish and rarer species, which are usually the target of conservation efforts. Also, the species composition, habituation levels, visitor loads in urban parks, type of human activities and how the temporal dynamics of visitors (daily and seasonal) may influence bird species' tolerance levels, population persistence and breeding success must be known.

Equally important are the recreational, educational, and productive functions of protected natural assets. Petrosillo et al. [41] noted that protected natural assets in urban areas can "support natural capital and consequently environmental security and human well-being. Natural protected areas can be considered part of the so-called 'critical social natural resource', representing natural areas that are of critical value largely as a result of their social value to local communities rather than any outstanding ecological or scientific value."

One of the most significant social benefits of nature protection is its role in educating and informing the public. This is particularly important for living in urban conditions as outlined in the results of different studies [32-33, 45]. Unfortunately, the practical confirmation of these findings cannot be found in our study except at the Jevremovac Botanical Garden.

CONCLUSIONS

The spatial disposition, functionality and survival of autochthonous ecosystems and abiotic rarities are increasingly threatened by anthropogenic pressure. Biotic and abiotic assets located in urban landscapes have the highest sensibility and are most threatened by human influence. Now in Belgrade, there are about 50 natural assets of different types that are protected by law and are under some management regime that guarantees their existence and functionality. These protected assets are of different types, structures, values, locations and significance. Through their protection, some level of compliance with the need to preserve the ecological balance and environmental protection and rational nature resource usage are fulfilled amid necessary urban development, society, infrastructure, and productive urban structure.

This report covers four protected assets situated on different locations from the centre to the edge of Belgrade city. It can be concluded that location does not influence the functions of these areas, which are legally protected by the Serbian law on nature protection.

It is expected that the structure of the protected areas will change through future Belgrade development. In the future, these changes should be studied, with special emphasis on the under-developed educational function for which there is high potential.

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Full Paper

Formulation and characterisation of valsartan proniosomes

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Abstract: Non-ionic surfactant vesicles of valsartan, an angiotensin II inhibitor, were prepared by coacervation phase separation method. The prepared systems were characterised for encapsulation efficiency, shape, size and in vitro drug release. Stability study was carried out to investigate the leaching of drug from the proniosomal system during storage. The results showed that valsartan in all the formulations was successfully entrapped and a substantial change in release rate and an alteration in the encapsulation efficiency of valsartan from proniosomes were observed upon varying the type of surfactant and cholesterol content. The encapsulation efficiency of proniosomes prepared with Span 60 was superior to that prepared with Span 40. A preparation with 9:2:9 ratio of Span 60, cholesterol and lecithin gave maximum encapsulation efficiency (71.50%) and release results (Q_{24h}= 75%) as compared to other compositions. Proniosomal formulations showed fairly high retention of valsartan inside the vesicles at refrigerated temperature (4-8^{o}C) up to 1 month.

Keywords: valsartan, proniosomes, niosomes, encapsulation efficiency, drug delivery

INTRODUCTION

Several classes of medications collectively referred to as antihypertensive drugs are available for treating hypertension. One such class is angiotensin II antagonists. Valsartan belongs to this category and is used as a choice for patients with heart failure who are unable to tolerate angiotensin converting enzyme (ACE) inhibitors in the management of hypertension. It is currently available as tablets and hard gelatin capsules in the market. The drug is rapidly absorbed following oral administration with a bioavailability of about 23% [1]. Valsartan is poorly soluble and the aqueous solubility is reported to be less than 1 mg/mL. It has a low molecular weight (435.5 g/mol) and melting point (116-117^{o}C) with a low partition coefficient (4.5) [1-2].

Some efforts have been made to enhance the solubility of valsartan to study its effect on the bioavailability of the drug. Valsartan/hydroxypropyl-β-cyclodextrin complex has been reported to significantly increase solubility and decrease the rate of valsartan degradation [3]. A Gelucire 50/13-based dispersed granule formulation has also been reported very recently [4]. The effect of various terpenes including a diterpene, forskolin (a putative penetration enhancer), on skin permeation of valsartan was investigated by Rizwan et al [5]. A self-microemulsifying drug delivery system (SMEDDS) has been developed by Dixit et al. [6] to enhance the diffusion rate and oral bioavailability of valsartan.

Vesicular carriers such as liposomes or niosomes have distinct advantages over conventional dosage forms because these particles can act as drug reservoirs. In recent years, non-ionic surfactant vesicles, also referred to as niosomes, have been studied as an alternative to conventional liposomes in drug delivery [7-9]**.** Compared to liposomes (phospholipid vesicles), they offer higher chemical stability, lower cost and greater choice of surfactants. However, even though niosomes exhibit good chemical stability during storage, there may be problems of physical stability in niosomal dispersions. Aqueous suspensions of niosomes may exhibit aggregation, fusion, leaking of entrapped drug or hydrolysis of encapsulated drug, thus limiting the shelf life of the dispersion [10].

The proniosomal approach minimises the above-mentioned problems, as it involves a dry product or a liquid crystalline gel that can be hydrated immediately before use [11-12]. Ease of transfer, distribution, measuring and storage makes proniosomes a versatile delivery system. Proniosomes are water-soluble carrier particles that are coated with surfactant and can be hydrated to form a niosomal dispersion immediately before use on brief agitation in hot aqueous media [13].

Various studies have demonstrated the successful use of proniosomes for delivery of antihypertensive drugs. For instance, Blazek-Welsh and Rhodes [14] prepared proniosomes of alprenolol hydrochloride using Span 60, dicetyl phosphate and cholesterol by slurry method. Maltodextrin was used as carrier that produced proniosomes with greater drug loading. Gupta et al. [15] investigated the potential of proniosomes as a transdermal drug delivery system for captopril. Proniosomes were found promising for transdermal delivery of this drug leading to a reduction in side effects. Thakur et al. [16] reported a proniosomal transdermal therapeutic system for losartan potassium .

The aim of this study is to investigate the feasibility of formulation of proniosomes of valsartan. Vesicles prepared were characterised by optical, scanning and transmission microscopy for vesicle formation and morphology. Drug encapsulation efficiency and release studies were carried out. Finally, a stability study of proniosomal formulations was also performed to investigate the leaking of the drug during storage.

MATERIALS AND METHODS

Chemicals and Reagents

Sorbitan monopalmitate (Span 40) and sorbitan monostearate (Span 60) were purchased from Shah Scientific, Mumbai, India. Soya lecithin (95%) was obtained as a gift sample from Ind Swift, Parwanu, India. Cholesterol (AR grade) was purchased from Central Drug House Pvt. Ltd, New Delhi, India. Absolute ethanol was purchased from Changshu Yangyuan Chemical, China.

Disodium hydrogen phosphate, sodium chloride and potassium dihydrogen phosphate (AR grades) were purchased from Qualigens Fine Chemicals Ltd. Mumbai, India for preparation of phosphate buffer saline (PBS, pH 7.4). Valsartan (99.8%) was obtained as a gift sample from Jubilant Organosys, Noida, India. Dialysis membrane (MW cut-off: 8000-10,000) was purchased from Himedia Laboratories Pvt. Ltd, Mumbai, India.

Equipment

Valsartan was estimated in samples by a UV-spectrophotometer (UV-1800, Shimadzu, Japan). A light microscope with digital camera (Coolpix S 220, Nikon, Japan), a scanning electron microscope (JSM-6100, Jeol, Japan) and a transmission electron microscope (Hitachi 7500, Canada) were used for morphological characterisation of vesicles. The pH of PBS was set using a pH meter (Max 962-P, Max Electronics, India). A centrifuge (REMI Group, Mumbai, India) and a sonicator (Power Sonic 410, India) were used.

Preparation of Proniosomes and Niosomes

Valsartan proniosomes were prepared by coacervation phase separation method (method modified from literature by Perrett et al. [17]). The composition of different proniosomal formulations is listed in Table 1. Briefly, drug (valsartan), surfactant (Span 40 or Span 60), lecithin and cholesterol were mixed with 2.5 ml of absolute ethanol in a wide-mouth glass tube. The tube was covered with a lid and warmed for 5 min at 65±3^{o}C in a water bath. PBS (1.6 ml) was added and the mixture was further warmed for about for 2 min so that a clear mixture was obtained. It was allowed to cool at room temperature until the dispersion was converted to a proniosomal gel. The gel was transformed to niosomes by hydrating with PBS (10 ml) at 80^{o}C by gentle mixing. The niosomes were sonicated twice for 30 seconds each with a sonicator and then used for further study [14].

Table 1. Composition of proniosomal formulations prepared

Proniosomal code	Drug (mg)	Span 60 (mg)	Span 40 (mg)	Lecithin (mg)	Cholesterol (mg)
PN 1	100	1800	-	1800	200
PN 2	100	1800	-	1800	400
PN 3	100	1800	-	900	200
PN 4	100	-	1800	1800	200
PN 5	100	-	1800	1800	400
PN 6	100	-	1800	900	200

Determination of Encapsulation Efficiency

Per cent encapsulation efficiency (EE) was determined by centrifugal method [18]. The proniosomal gel was converted to a niosomal dispersion, which was centrifuged (18000 rpm) for 40 min at 5^{o}C in order to separate unentrapped drug. The supernatant was taken and diluted with PBS. The drug concentration in the resulting solution was assayed spectrophotometrically at 250 nm. The

percentage of drug encapsulation was calculated by the following: EE (%) = $[(C_t - C_f)/ C_t] \times 100$, where C_t is the concentration of total drug and C_f is the concentration of unentrapped drug.

Characterisation of Valsartan Proniosomes

Optical microscopy and vesicle size determination

A drop of niosomal dispersion prepared from proniosomes was spread on a glass slide and examined for the vesicle structure and presence of insoluble drug crystals under the light microscope with varied magnification power. Photomicrographs were taken for niosomes using a digital camera with 6X optical 200 m.

The proniosomal gel (100 mg) was hydrated with PBS (10 ml) in a small test tube by manual shaking for 5 min and the resulting niosomes were observed under optical microscope at 100 X magnification. The average size of vesicles was measured using calibrated ocular and stage micrometer in the microscope.

Scanning electron microscopy

The niosomes formed from the hydration of proniosomal gel were mounted on an aluminum stub with double-sided adhesive carbon tape. The vesicles were then sputter-coated with gold/palladium using a vacuum evaporator and examined with the scanning electron microscope equipped with a digital camera at 25kV accelerating voltage [19].

Transmission electron microscopy

The morphology of the hydrated niosomal dispersions prepared from proniosomes was also determined by transmission electron microscopy. A drop of niosomal dispersion was applied to a carbon-coated 300-mesh copper grid and left to adhere on the carbon substrate for about 1 min. The remaining dispersion was removed by a piece of filter paper. A drop of 2% aqueous solution of uranyl acetate was applied for 35 seconds and again the solution in excess was removed by the tip of filter paper. The sample was air-dried and observed under the transmission electron microscope at 90kV [13].

In vitro release study

In vitro release pattern of niosomal suspension prepared from proniosomes (as prescribed above) was carried out using a dialysis bag (high-media dialysis membrane, 8000-10,000 MW cut-off) as a donor compartment [20]. An accurately measured amount of valsartan niosomes, equivalent to 20 mg valsartan, were taken in the dialysis membrane and placed in a beaker containing 75 ml of PBS, which acted as receptor compartment. Previously, the dialysis membrane was soaked in warm water for 10 min and both ends were sealed with closure clips after adding the niosomal preparation. The beaker was placed over a magnetic stirrer (100 rpm) and maintained at 37±1^{o}C. At predetermined time intervals during 24 hr, aliquots (1ml) were withdrawn and replaced with fresh buffer. The sink condition was maintained throughout the experiment. The withdrawn samples were appropriately diluted and analysed for drug content spectrophotometrically at 250 nm using PBS as blank. The results were the mean values of three runs.

Stability of valsartan proniosomes

A physical stability test was carried out to investigate the leaching of drug from proniosomes. The proniosomal samples were sealed in 20-ml glass vials and stored at refrigeration temperature (4-8^{o}C) and at 37^{o}C for one month. The EE of all the samples was determined in the same manner as prescribed previously after one month [21].

Statistical analysis

Statistical analyses of % EE and in vitro release of the proniosomal formulations were performed using one-way analysis of variance (1-way ANOVA) and paired t-test respectively (Graph Pad, version 3.0, San Diego, CF). The level of significance was taken at p value < 0.05.

RESULTS AND DISCUSSION

Optical Microscopy and Vesicle Size Determination

The photomicrographs of hydrated PN1 and PN2 proniosomal formulations (Table 1), composed of Span 60 and cholesterol in 9:1 and 4.5:1 ratios, are shown in Figure 2 and Figure 3 respectively. The photographs reveal that the niosomes are unilamellar vesicles having spherical shape and no aggregation or agglomeration is observed. Apparently, PN1 niosomal formulation gives vesicles of larger sizes.

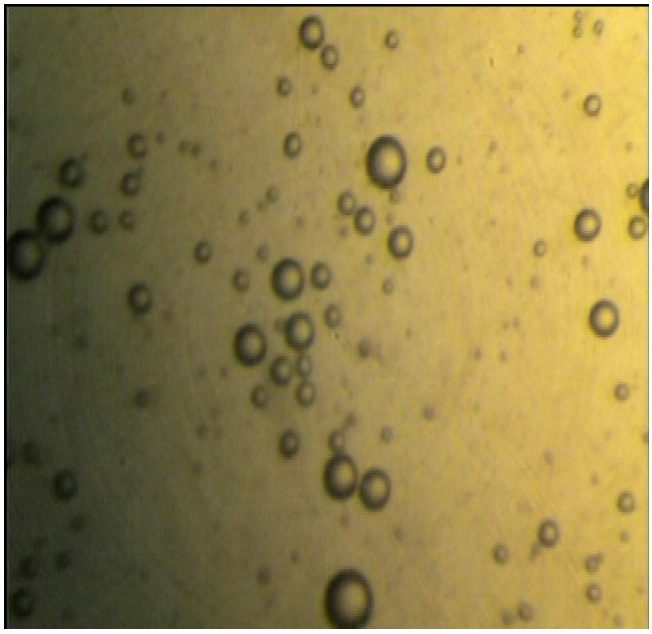

Figure 2. Photomicrograph of hydrated PN1 proniosomal formulation

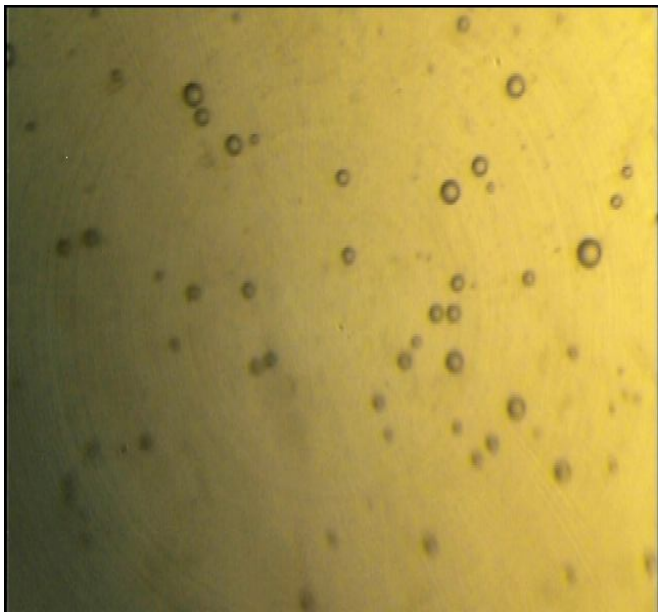

Figure 3. Photomicrograph of hydrated PN2 proniosomal formulation

The mean particle sizes of all the hydrated proniosomal formulations are shown in Table 2, which shows that the niosomes composed of Span 60 are larger in sizes than those obtained using Span 40. Span 60 has longer saturated alkyl chains compared to Span 40 [22] and it was reported that surfactants with larger alkyl chains generally give larger vesicles [23]. This seems to account for the high EE obtained with Span 60 proniosomes. Moreover, increasing cholesterol content or reducing lecithin content contributed to an increase in hydrophobicity with consequent reduction in vesicle sizes as listed in Table 2.

Scanning and Transmission Electron Microscopy

Results of scanning and transmission electron microscopic study of niosomes prepared from PN1 and PN2 proniosomal formulations are shown in Figures 4-7. Most of the vesicles are well identified, spherical and discreet with sharp boundaries having large internal aqueous space.

Encapsulation Efficiency

The EE of all proniosomal formulations are reported in Table 2. From the table, increase in cholesterol, one of the common additives for preparing stable proniosomes, is seen to increase EE of valsartan. An increase in cholesterol content has also been found to result in increase in microviscosity of the membrane leading to more rigidity of the bilayers [23]. Cholesterol seems to have an ability to cement the leaking space in a bilayer membrane [24].

All Span-type surfactants have the same head group with different alkyl chains. Increase in the alkyl chain length has been found to lead to a higher EE [25]. Span 60 has a longer saturated alkyl chain (C_{16}) compared to that of Span 40 (C_{14}). A larger alkyl chain lowers the HLB value of a surfactant and this tends to increase EE of the drug [22]. It is clear that the PN2 formula, which was

composed of Span 60, cholesterol and lecithin in a 9:2:9 ratio, seems to be the most suitable for an efficient encapsulation of valsartan as it exhibits the highest EE (71.5 %).

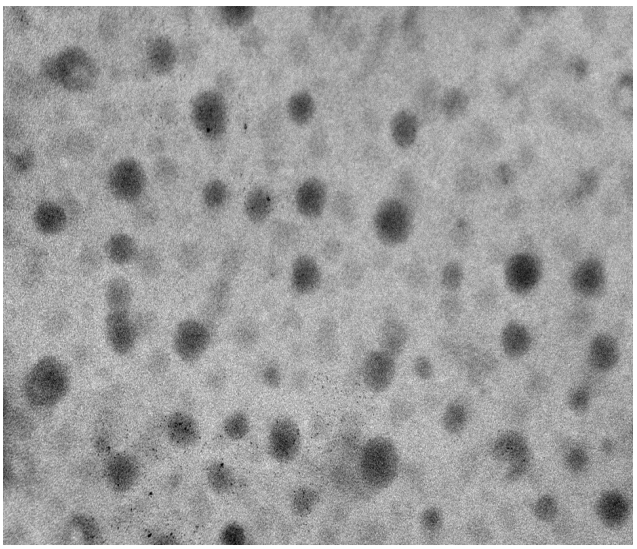

Figure 4. Transmission electron micrograph of hydrated PN1 proniosomal formulation (at 90 kV with magnification 100,000 X)

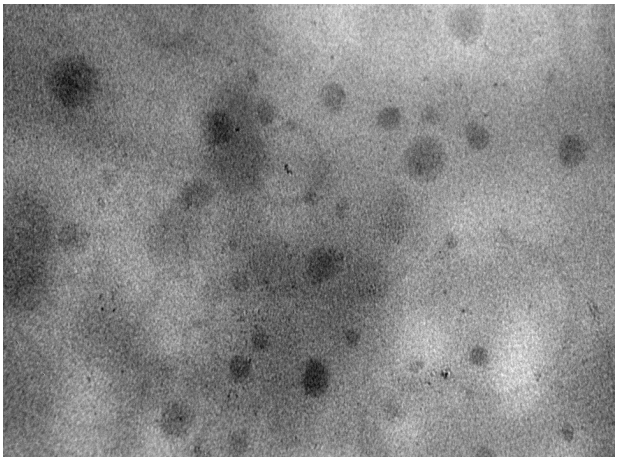

Figure 5. Transmission electron micrograph of hydrated PN2 proniosomal formulation (at 90 kV with magnification 100,000 X)

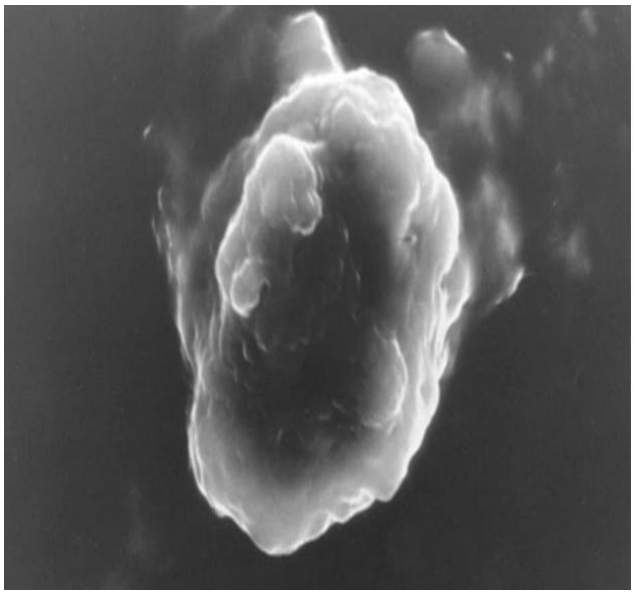

Figure 6. Scanning electron image of hydrated PN1 proniosomal formulation (at 25 kV with magnification 2,000 X)

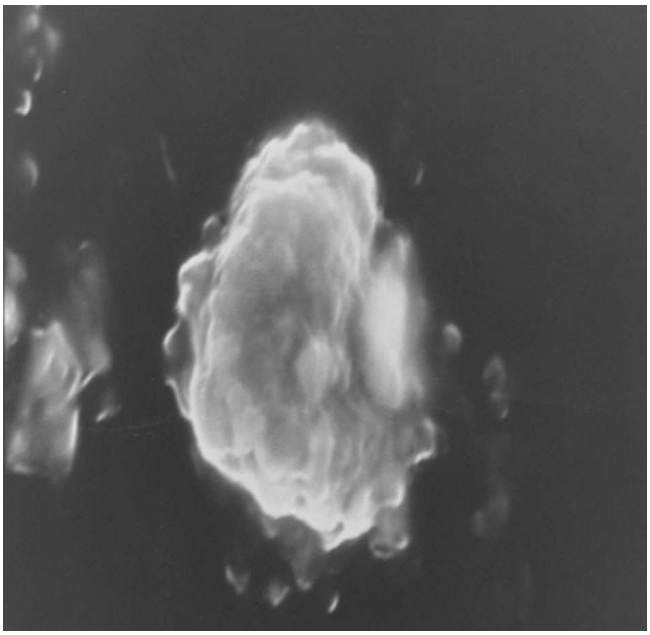

Figure 7. Scanning electron image of hydrated PN2 proniosomal formulation (at 25 kV with magnification 2,000 X)

Table 2. EE and vesicle size of hydrated proniosomes

Formulation	Surfactant	Lecithin (mg)	Cholesterol (mg)	EE (%) ± S.D.	Vesicle size (μm) ± S.D.
PN 1	Span 60	1800	200	66.97 ± 2.35	5.26 ± 0.46
PN 2	Span 60	1800	400	71.47 ± 1.61	4.19 ± 0.082
PN 3	Span 60	900	200	69.47 ± 1.16	3.61 ± 0.105
PN 4	Span 40	1800	200	49.43 ± 2.11	3.36 ± 0.198
PN 5	Span 40	1800	400	51.83 ± 1.59	3.03 ± 0.066
PN 6	Span 40	900	200	43.34 ± 4.13	2.80 ± 0.025

In Vitro Release Study

The dialysis method was used to investigate the in vitro valsartan release from niosomes. Results are shown in Figure 8. The percentage of the drug released after 24 hr from the niosomal vesicles are shown in Table 3. Formulations which have higher cholesterol content (PN2 and PN5) are seen to have less drug release over a period of 24 hr. Hence, increase in cholesterol ratio seems to result in a more intact bilayer and consequent reduction in permeability.

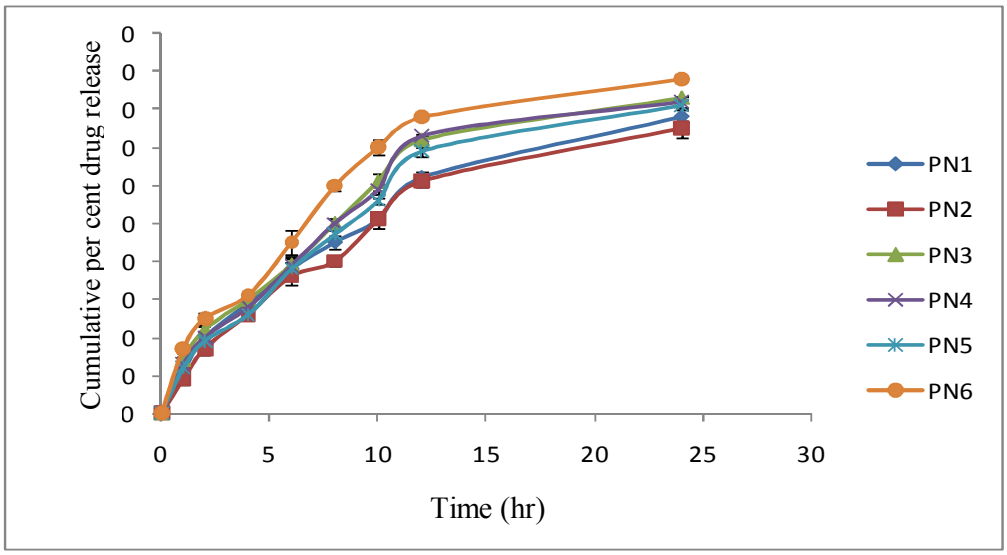

Figure 8. In vitro drug release of niosomes prepared from various proniosomal formulations

Table 3. In vitro release of valsartan from niosomes prepared from various proniosomal formulations after 24 hr

Formulation	Per cent release ± S.D.
PN 1	78.05 ± 1.87
PN 2	74.96 ± 2.23
PN 3	83.02 ± 0.62
PN 4	82.08 ± 1.37
PN 5	81.01 ± 2.31
PN 6	88.09 ± 0.34

By inspection of the data, it is also evident that proniosomal formulation with less amount of lecithin gives a faster rate of drug release, probably owing to disruption of structure of vesicles having a reduced amount of lecithin. However, this increase in release rate was found to be insignificant ($p>0.05$) in both cases. Among all formulations, those with Span 40 showed statistically significant ($p<0.05$) increase in release cf. Span 60, keeping all other additives the same. The large vesicle size of Span 60 formulations also tends to act as barrier to the drug release thereby reducing it. It is to be noted that the in vitro release results are consistent with those of EE; PN2 proniosomes with highest EE (71.47%) show lowest drug release (74.96%) after 24 hr. Similar results were obtained by Guinedi et al. [22].

Mathematical models are commonly used to predict the release mechanism and compare release profile. For all the formulations (PN1 to PN6), the cumulative per cent drug release vs time (zero order), the cumulative per cent drug release vs square root of time (Higuchi plot), and log cumulative per cent drug remaining vs time (first order) were plotted separately (not shown here). In each case, r^2 value was calculated from the graph and reported in Table 4. Considering the determination coefficients, Higuchi model was found to fit the release data best. This demonstrates that valsartan molecules were dispersed in the proniosomes matrix and there was no interaction between the drug and proniosomes material. The first order release model fitting of the release data shows that the release rate was concentration- dependent. It is therefore concluded that the drug was released from proniosomes by a diffusion-controlled mechanism. The results are in good consistency with the experimental results observed by Guinedi et al. [22].

Stability Studies

Physical stability of proniosomal formulations were studied for a period of one month. The EE were determined for all proniosomal formulations stored at 4-8^{o}C and 37^{o}C as shown in Figure 9, which indicates insignificant decrease in EE of proniosomes stored at 4-8^{o}C: approximately 90% of valsartan was retained in all proniosomal formulations after the one-month period. Thus, both Span 40 and Span 60 proniosomes of valsartan seemed to exhibit good stability at low temperature.

Table 4. Kinetic analysis release data of valsartan proniosomal formulations

Formulation	Zero order (r^2)	Higuchi model (r^2)	First order (r^2)
PN 1	0.886	0.986	0.982
PN 2	0.885	0.974	0.969
PN 3	0.852	0.965	0.958
PN 4	0.849	0.958	0.944
PN 5	0.871	0.966	0.962
PN 6	0.813	0.953	0.951

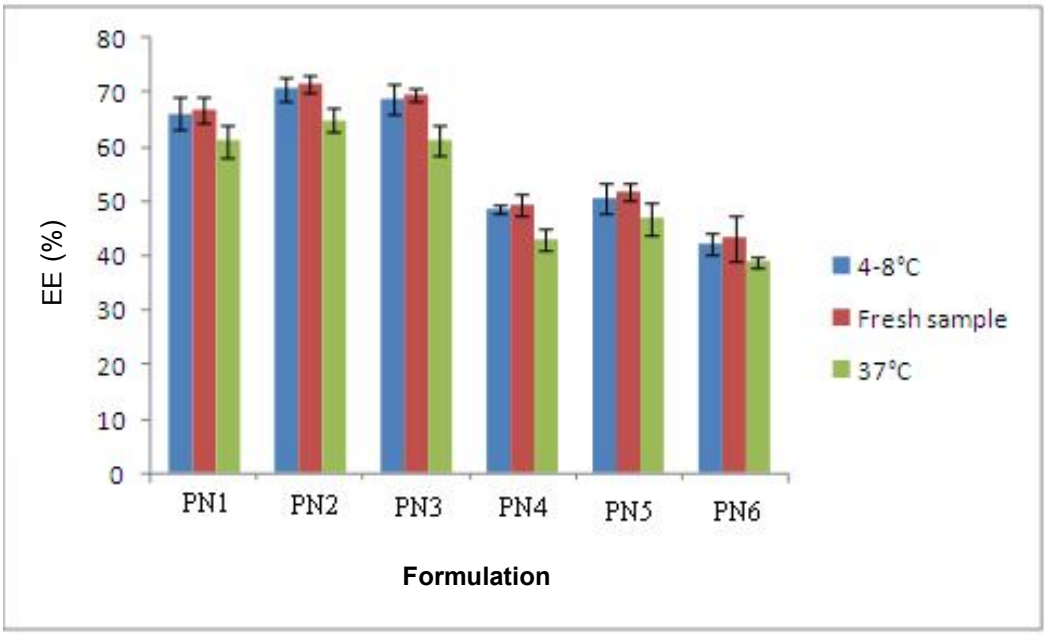

Figure 9. Comparison of EE of valsartan proniosomal formulations after one month

CONCLUSIONS

Using coacervation phase separation method, valsartan has been successfully incorporated in proniosomal formulations which can be potentially useful for delivery of this drug. Results of the present work have shown that surfactant type and content of cholesterol and lecithin affect the encapsulation efficiency and drug release rate from proniosomes. A maximum encapsulation efficiency of 71% and drug release of 88% after 24 hr have been attained. Encapsulation efficiency of proniosomes formed by Span 60 was observed to be higher compared to that obtained with Span 40. Valsartan proniosomes were also found to be quite stable at 4-8^{o}C over a one-month period. This work has established the foundation for future study on the potential of valsartan-loaded niosomes for a transdermal delivery system.

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Communication

Salinity triggers proline synthesis in peanut leaves

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Abstract: To investigate the magnitude of proline accumulation under different salinity levels, a salinity-imposed experiment was conducted. The peanut seedlings were cultured hydroponically for 7 days before imposing salinity and then grown further for another 7 days under salinity-loaded condition. Results showed that shoot dry matter, relative water content, chlorophyll and K^+ decreased significantly with increasing salinity. In contrast, Na^+, hydrogen peroxide and proline increased with increasing salinity level. Free proline content increased abruptly at medium and high salinity levels. Interestingly, the lowest level of dry matter (an indicator of tolerance/sensitivity) accompanied by the highest accumulation of proline at 200 mM NaCl puts a question mark on the well-documented role of proline in relation to salinity tolerance. The underlying mechanism is discussed in detail in the current study.

Keywords: salinity, proline biosynthesis, peanut leaves

INTRODUCTION

Salinity is one of the most important environmental stresses which severely limits plant growth and productivity worldwide [1-2]. The problem is ever increasing because of irrational human acts causing secondary salinisation [3] and also because of global warming with consequent rise in sea level and increase in tidal surges, particularly in coastal areas [4]. In Bangladesh, more than 1 million hectares of the coastal areas have been seriously affected by salinity [5], which is considered as one of the major problems of crop production in Bangladesh.

Salinity exerts its undesirable effects through osmotic inhibition, ionic toxicity and also by disturbing the uptake and translocation of nutritional ions [6]. Now it is widely accepted that abscisic-acid-mediated root signals limit the availability of water to the plant cells, which leads to

slower plant growth during salinity stress [7-9]. This is known as the 'osmotic effect' of salinity [10]. This effect can disturb the physiological and biochemical functions of the plant cells, leading finally to cell death [11]. So osmotic balance is certainly crucial for the survival of a plant under salinity-stressed condition. Under various environmental stresses, plant cells have experienced the accumulation of some organic solutes such as sucrose [12], glycinebetaine [13], mannitol [14], trehalose [15-16] and proline [17-18], and these organic solutes contribute to the maintenance of turgor. Plants under salinity stress also accumulate a number of metabolites, which are termed compatible solutes because they do not interfere with the plant's metabolism even at molar concentrations [19]**.** The accumulation of such compatible osmolytes involved in osmoregulation allows additional water to be taken up from the environment, thus buffering the immediate effect of water shortage within the plant [20].

Among the accumulation of compatible osmolytes, that of proline is one of the most frequently reported modifications induced by water deficit as well as salinity stress in plants. Several functions are proposed for the accumulation of proline in tissues exposed to salinity stress: osmotic adjustment [21], C and N reserves for growth after stress relief [22-23], detoxification of excess ammonia [24], stabilisation of proteins and membranes [25], protection of macromolecules from denaturation [26], osmoprotection [27], free radical scavenging [28], antioxidation [29] and regulation of cytosolic acidity [30]. In addition, proline biosynthesis may be associated with the production of $NADP^+$ for the stimulation of the pentose phosphate pathway [31]. Now it is well documented that proline plays a predominant role in protecting plants from osmotic stress. In the present study, we aim to investigate the magnitude of proline accumulation under different levels of salinity in peanut seedlings.

MATERIALS AND METHODS

Plant Material and Treatments

Seeds of groundnut (*Arachis hypogea* L., genotype Dhaka-1) were obtained from the plant breeding division of Bangladesh Institute of Nuclear Agriculture (BINA). The seeds were surface sterilised with ethanol for 3 minutes. The surface-sterilised seeds were rinsed thoroughly with distilled water and placed on water-soaked filter paper at 25^{o}C to germinate. After germination, healthy and vigorous seedlings with uniform roots were selected and transferred to perforated plastic sieves, each of which contained 4 seedlings. The seedlings were grown hydroponically in modified half-strength Hoagland solution [5] at 25^{o}C and 60% relative humidity with a photoperiod of 16 hr (700 μmol m^{-2} s^{-1}). The seedlings were cultured for 7 days at pH 6.5 in the growth chamber under the control environment as described by Rahman et al [5]. The pH of the nutrient solution was adjusted to 6.5 using H_2SO_4 and NaOH. A salinity-imposed experiment was then conducted in full-strength Hoagland solution supplemented with 4 concentrations of salt (0, 50, 100 and 200 mM of NaCl) for 7 days. The experimental containers were laid out in completely randomised design (CRD) with 4 replicates, giving a total of 16 plots. Then the plants were harvested and seedling growth was determined by measuring the length and fresh weight of root and shoot. Plant materials were oven-dried (70^{o}C for 24 hr) and dry weights were recorded.

Relative Water Content

A pre-dawn leaflet sample was taken from three plants for each replicate in each treatment on the 7th day after salinisation and its fresh weight immediately recorded. The leaf sample was then incubated in deionised water for 4 hr as described by Sairam et al. [32], after which the turgid weight of the leaf sample was taken. The leaf sample was then packed in a butter paper bag and oven-dried at 65°C for 48 hr and the dry weight of the sample was taken. The relative water content (RWC) was estimated as follows:

$$RWC = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Turgid weight} - \text{Dry weight}} \times 100$$

Proline Determination

Free proline was determined according to the method of Bates et al. [33] with slight modification. Briefly, fresh leaf sample (500 mg) was homogenised in 5 ml of 3% sulphosalicylic acid by a mortar and pestle and then centrifuged at 18000 g for 10 min to remove cell debris. The resulting extract (2 ml) was taken in a test-tube and glacial acetic acid (2 ml) and ninhydrin reagent (2 ml) were added. The reaction mixture was boiled in a water bath for 60 min. After cooling of the tube in ice, toluene (6 ml) was added and mixed thoroughly. Then the upper toluene phase was separated into a glass cuvette and free proline was quantified spectrophotometrically at 520 nm. Proline concentration was calculated from proline standard (0-50 μg/ml) treated in an identical manner.

H_2O_2 Determination

H_2O_2 concentration in the leaves of peanut genotype was measured spectrophotometrically as described by Alexieva et al. [34]. Freshly harvested leaves (0.5 g) were crushed into a fine powder in a mortar under liquid N_2. Then it was centrifuged at 12000 g for 10 min at 4°C and the supernatant was collected for determination of H_2O_2. The reaction mixture consisted of 0.5 ml of 0.1% trichloroacetic acid (TCA) containing the leaf extract supernatant, 0.5 ml of 100 mM potassium phosphate buffer and 2 ml of KI reagent (1 M KI in distilled H_2O). The blank consisted of 0.1% TCA instead of the leaf extract. The reaction was allowed to run for 1 hr in the dark and the absorbance was measured at 390 nm. The amount of H_2O_2 was calculated using a standard graph of known concentrations.

Chlorophyll Determination

Fully expanded leaves (3rd and 4th from the top, photosynthetically active) were sampled for chlorophyll determination. Fresh leaf samples (0.5g) were homogenised in cold 80% acetone (4 ml) in a cold mortar and centrifuged at 3000 rpm for 10 min at 25°C. Then the supernatant was collected and the volume made up to 20 ml with 80% acetone. The absorbance reading was taken at 645 nm with a UV-VIS spectrophotometer and the chlorophyll content expressed as mg ml^{-1} [35].

Na^+ and K^+ Content

About 0.3 g dry and ground leaves was placed in a digestion tube and 2.5 ml of digestion mixture (H_2SO_4 + $HClO_4$) was added [5]. After mixing, the tube was allowed to stand for 2 hr, then placed in a heating block and heated for 2 hr at 100°C. After cooling, three 1-ml aliquots of 30% H_2O_2 were added, the content of the tube being thoroughly mixed after each addition. The tube was then placed in an aluminium block and heated to 330°C (just below the boiling point of the digestion mixture) for about 2 hr. The cooled, clear, digested mixture was diluted to 20 ml with deionised water and filtered, and aliquots were taken for analysis. Na^+ and K^+ ion concentrations in the extract were estimated by flame photometry and expressed as % dry matter (DM).

Statistical Analysis

All data were subjected to analysis of variance by CRD method and data were expressed as mean ± SE obtained from four independent experiments. Duncan's multiple range test was applied to compare the treatment means. P value ≤ 0.05 was considered as significant.

RESULTS

Figure 1 shows a gradual decrease in shoot DM with increasing salt concentration. High salinity stress (200 mM NaCl) resulted in 50% decrease in shoot DM compared to control. RWC in the peanut leaves was also found to decrease with increasing salt concentration (Figure 2a). At 200 mM salinity a decrease of 13% compared to control can be observed.

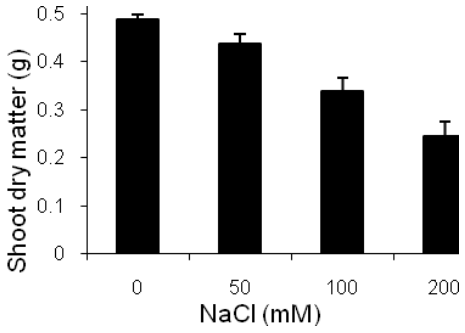

Figure 1. Effect of salinity on shoot dry matter in peanut seedlings. Vertical bars indicate means ± S.E. (n=4).

From Figure 2b, the free proline content in peanut leaves can be observed to dramatically increase with increasing salinity: a 2.5-, 10- and 18-fold increase in proline accumulation at 50, 100 and 200 mM NaCl respectively. The free proline content increased slowly at low salinity and rapidly at medium and high salinity. In response to salinity, H_2O_2 content also increased significantly with the level of salinity (Figure 2c). It increased more than threefold when the young plants were exposed to 200 mM NaCl. Conversely, the total chlorophyll content in the leaves decreased 50% from control at 200 mM NaCl (Figure 2d), indicating that the salt present in the culture solution might be involved in the damage of the chloroplasts.

When peanut seedlings were grown on hydroponic culture solutions containing various concentrations of NaCl, the sodium concentration in the leaves was always higher than that in the

control (Table 1) and was found to increase with increasing salinity level. A reverse trend was observed for K^+ concentration. As a consequence, the K^+/Na^+ ratio decreased with increasing concentration of NaCl in the culture solution.

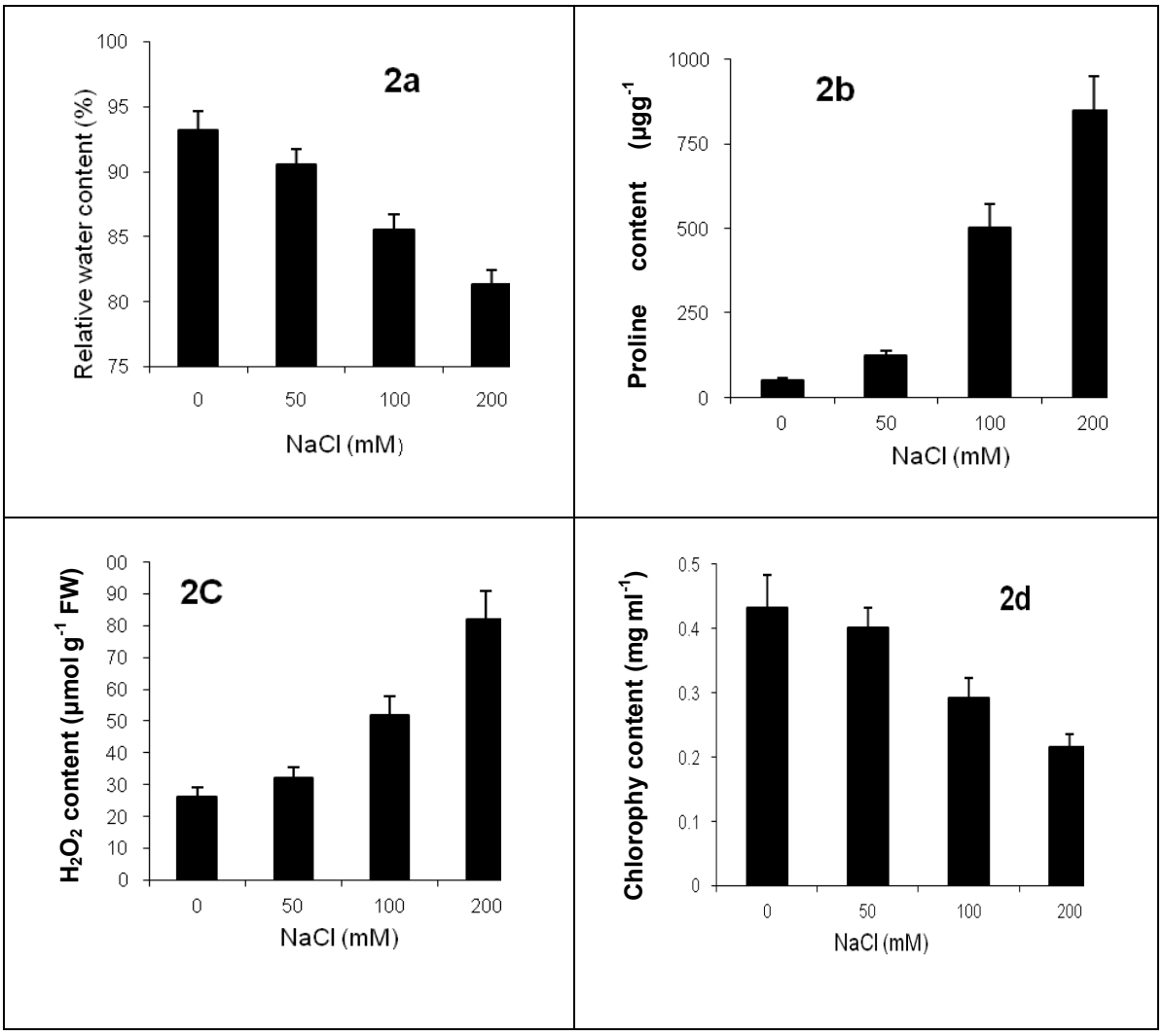

Figure 2. Effect of salinity on (a) RWC (b) proline content, (c) H_2O_2 content and (d) chlorophyll content in peanut seedlings. Vertical bars indicate means ± S.E. (n=4).

Table 1. Sodium and potassium content (% of DM) in leaves of peanut seedlings. Each value represents the mean ± S.E obtained from four independent experiments; $P \leq 0.05$.

Treatment (mM NaCl)	Na^+ content (% DM)	K^+ content (% DM)
0	0.23 ± 0.01	6.99 ± 0.08
50	0.35 ± 0.03	6.40 ± 0.06
100	9.55 ± 0.08	1.75 ± 0.24
200	13.33 ± 0.06	0.63 ± 0.01

DISCUSSION

Proline accumulation, a common metabolic response of plants subjected to salinity stress, is considered to be involved in stress-tolerance mechanisms [20]. In the present study, a significant increase in proline content was found in peanut seedlings after 7 days of exposure to NaCl stress (Figure 2b). The results also revealed that the magnitude of proline accumulation was positively (R^2=0.98, P≤0.05) associated with the concentration of NaCl in the culture solution. These results are consistent with the findings of some earlier studies [36-37]. One distinctive feature of most plants growing in saline environments is the accumulation of proline [1] and it has been inferred that there may be a relationship between cellular proline level and cell turgidity via osmotic adjustment [23, 38]. Interestingly, such beneficial effect of elevated proline level was not reflected in the maintenance of relative water content in peanut leaves in the present study. The results could be interpreted as follows. Firstly, salinity stress limits the uptake of CO_2 [8-9], resulting in decreasing carbon reduction by Calvin cycle [39], which leads to non-availability of $NADP^+$ for acceptance of electrons during photosynthesis. In this situation, photosynthetic reducing power, $NADPH_2$, is used for proline biosynthesis and consequently $NADP^+$ is regenerated [40]. These reactions are summarised in Figure 3. The excess accumulation of proline may therefore be a result of metabolic changes induced by high salinity. The present results also agrees with the observations of Delauney and Verma [41], who stated that excess proline accumulation in response to high salinity functions by other than osmotic adjustment.

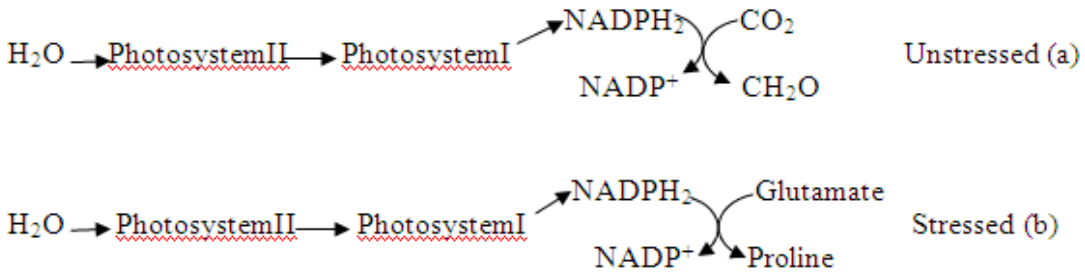

Figure 3. Schematic illustration of electron flow in : (a) normal physiological condition and (b) high-salinity stress condition, which can lead to inhibition of photosynthesis by diverting the flow of photoreductant (e^-) from the CO_2 reducing system to proline biosynthesis, leading to reduced growth and productivity of the plants.

The low shoot DM (an indicator of salt sensitivity) along with the high proline content also suggests that the increase in proline concentration may not be associated with salinity tolerance, which agrees with some previous studies [9, 42]. Thus, the maximal accumulation of proline might have occurred when plants were exposed to excessive salinity which might damage them fatally. Also, other osmolytes other than proline might be involved in leaf water content or cell turgidity. This was also reflected in our current investigation. The concentration of inorganic osmoticum (K^+) in the leaf tissues might be associated (R^2=0.97, P≤0.05) with relative water content in the leaves (Figure 2a) and actively contribute to turgor maintenance of the cells [43]. Further investigation is needed to clarify their relative importance in turgor maintenance.

The low DM yield in high salinity level may be explained by excess accumulation of toxic ions, particularly Na^+ with concomitant reduction of K^+ ions (Table 1), an important osmolyte in the cellular environment. The degree of inhibition of K^+ uptake is associated with salinity levels in the culture solution (Table 1). This is reasonable as high Na^+ concentration negatively affects K^+ acquisition due to similar physicochemical properties of Na^+ and K^+ [44]. Moreover, low DM yield and low chlorophyll content (Figure 2d) in highly salinised leaves might be related to the elevated levels of H_2O_2 (Figure 2c), which was also stimulated by salinity [36], resulting in serious photodamage of chlorophyll [45-46].

Taken all together, it may be concluded that in the present study proline accumulation was a result of photosynthetic impairments or metabolic changes induced by high salinity whereas its function in maintaining cell turgidity/relative water content was not so strong.

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