UNIVERSITI MALAYA

INAUGURAL LECTURE

"Immobilized Enzymes and Cells, Microbial Indicators: Application and Technology"

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February 20, 2004

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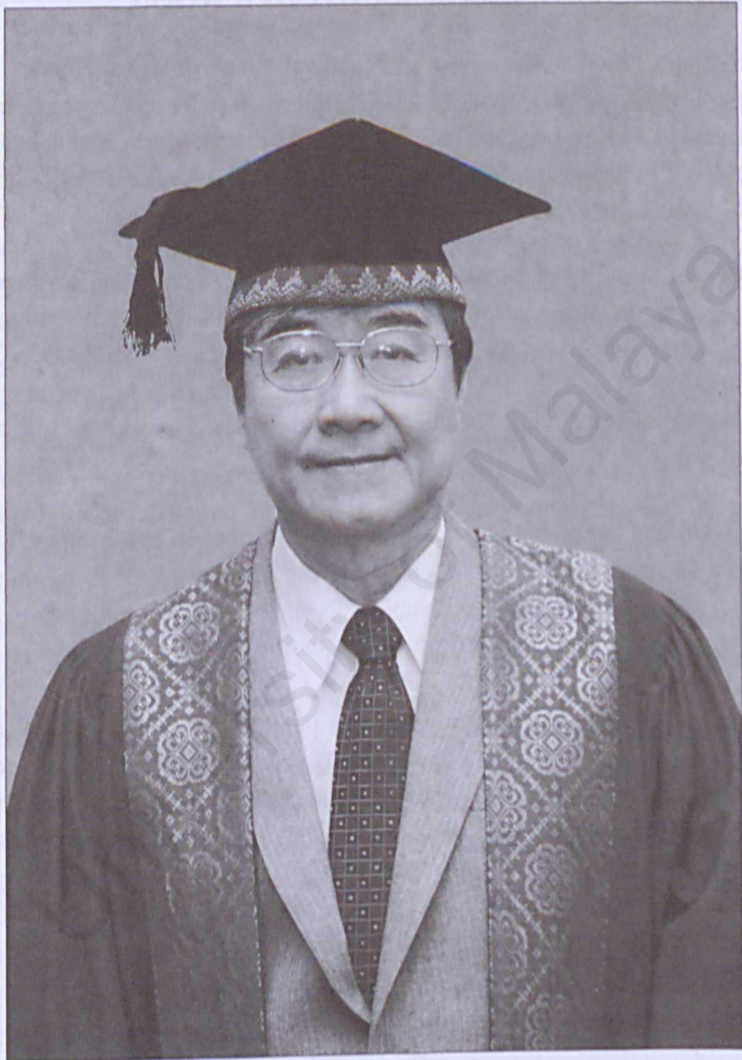

PROFESSOR WANG CHEE WOON

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Curriculum Vitae

PROFESSOR WANG CHEE WOON

Professor Wang Chee Woon was born in Penang and received his secondary education from Chung Ling High School and Methodist Boys School, Penang. He graduated with a B.Sc.(Hons.) and Ph.D. from the University of Malaya, Kuala Lumpur. He joined the Department of Biochemistry, University of Malaya as a Lecturer in 1978. He was promoted to Associate Professor in 1986 and to Professor in 2000.

Prof. Wang was a visiting Commonwealth fellow in the University of Birmingham from 1983-84, working on large scale production of biocatalysts. He was a visiting associate professor in the Biotechnology Research Centre, University of Waterloo, Ontario, Canada from 1989-1990. In 1996-1997, he was a visiting scientist in Massachusetts General Hospital/Harvard Medical School, Boston, working on molecular pathogenesis.

Professor Wang's main research interests centralize on applied enzymology, working on immobilized enzymes and cells, especially the use of biocatalysts in bio-modification of oils and fats. His main consultancy tasks are on water quality as well as the development of criteria and standard in water quality.

Professor Wang was the Hon. Gen. Secretary of the Malaysian Biochemical Society and also the Editor of Newsletters published by the Federation of Asian and Oceanic Biochemists (FAOB). He was one of the water resource specialists for the International Development Research Council (IDRC), Ottawa, Canada. He had served as a member of various Working Groups in the Asian-Australian Programme on Waste Utilization and Asian-Canadian Programme on Marine Sciences. Since 2001, he has served as an expert on biological research centres for the Organisation for Economic Co-operation and Development (OECD) in Paris, France.

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Immobilized Enzymes and Cells, Microbial Indicators: Application and Technology

Synopsis

Enzymes are biocatalysts of biological systems and are mainly globular proteins. They are remarkable molecular devices that determine the patterns of various chemical transformations. The most important hallmarks of enzymes are their catalytic abilities and specificities. The action of hydrolytic enzymes or hydrolases is important in food, pharmaceutical and cosmetic industries. The cost of enzymes can contribute significantly to the overall economy of an enzyme-catalysed product. Immobilization of an enzyme is therefore a technological process to reduce overall cost. Enzyme immobilization is a technological process whereby an enzyme has been confined or localized so that it can be reused continuously. In certain cases, immobilized enzymes may retain their activity longer than those in solution. Furthermore, the efficiency of catalysis for the multi-step conversion can be increased as the enzyme is immobilized in position near or next to other enzymes in a multi-enzymatic complex system.

The application of immobilized cell biocatalysis is also important in food and pharmaceutical industries. The salient feature of immobilized cell systems is the use of some confining or binding structure to constrain the cells in a particular region of the bioreactor. One main reason of cell immobilization is attainment of higher cell densities than in suspended cell systems. Cell immobilization offers continuous processing without organism wash-out and allows continuous separation of product and removal of reaction inhibitors. The immobilized cell containing one or more enzymes may essentially serve as immobilized enzyme biocatalyst, with the entire cell used in order to minimize treatment and processing costs.

Microbial indicators in treated and raw water sources have gained much attention recently. It is not practical to routinely monitor water for the presence of specific pathogenic organisms as the numerous types of organisms would require specific methods of detection and these pathogens are often present in small numbers and only intermittently. Any organism that occurs naturally in the faeces and not elsewhere is a potential indicator of faeces. Therefore, the public health regulations that limit the numbers of these organisms in potable and recreational waters provide an important degree of protection to consumers of these waters. The bacteria which have gained some acceptance as indicators are the coliform, faecal coliform, *Escherichia coli*,

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enterococci and somatic coliphages. We have reported a significant correlation between coliphage enumerated by the APHA standard method for coliphage detection and coliforms enumerated by conventional techniques, such as the multiple tube MPN (most probable number), and m-FC (faecal coliforms) methods. Linear regression analysis of their $\log_{10}$ transformed data showed significant correlation between coliforms and coliphages for surface water ($p<0.0001$) and well water ($p<0.001$). We have also reported the construction of coliphage field kit to be used in rural areas. The field kit was used for monitoring microbial water quality in wells and other water sources. A classification system on water quality based on coliphages was also proposed.

Introduction

What are enzymes?
What are immobilized enzymes?
Application of immobilized enzymes
What are immobilized cells?
Immobilized cells application and technology
Microbial indicator and water quality
Future directions and initiatives

What are Enzymes ?

Enzymes are biocatalysts of biological systems and are mainly globular proteins. They are remarkable molecular devices that determine the patterns of various chemical transformations. The most important hallmarks of enzymes are their catalytic ability and specificity (Berg *et al.*, 2002). The first successful isolation of a pure enzyme was achieved in 1926 by Sumner (Bailey and Ollis, 1986). The number of known enzymes has increased rapidly and the current total known enzymes is well in excess of 1,500.

In any enzyme-catalyzed bioprocess, the cost of enzyme is a major significant factor for consideration. The cost of enzymes varied from US$50-1,000/kg in 1984 (Dwyer,1984). The figures are certainly much higher today. Therefore, reusability of the enzyme is always a preferred direction. The use of immobilized enzymes is one of the ways to reduce the cost of production.

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What are Immobilized Enzymes?

An immobilized enzyme is one whose movement in space has been restricted either completely or to a small limited region (Wang *et al.*, 1979). Enzymes are often immobilized onto solid supports to increase their temperature or thermal and operational stability and recoverability. The methods of immobilization have been subdivided into four main groups. The first division is based on whether immobilization has been achieved primarily by entrapment in a limited space or by binding to a support or carrier material. The entrapped enzymes are further sub-divided into encapsulation and in matrix entrapment. The binding of enzymes to support materials may be by physical adsorption or by chemical or covalent binding.

Absorbed Enzymes

As I have mentioned earlier, absorbed enzymes are enzymes bound to the support materials by physical adsorption. The main support materials are alumina, bentonite, cellulose, sephadex, silica gel and glass. I would like to show you the procedure for preparing immobilized lipase by adsorption. The silica gel was first suspended in an aqueous *Humicola* fungal lipase solution. The mixture was stirred 30 min after which it was filtered. The immobilized enzyme was washed with cold water, air-dried and ready to use (Goh *et al.*, 1993).

Immobilized Lipases

The main advantages of using immobilized lipases or enzymes are specificity for different types of substrates; high yields; mild operating conditions; easy recovery; reusability of biocatalyst and low level of contamination (Langrand *et al.*, 1988).
By using a lipase-catalyzed reaction such as transesterification of triglycerides or oils, we have shown that the immobilized lipase is 1,3-specific. If the two fatty acid chains at positions 1 and 3 of a triglyceride are hydrolyzed, leaving a monoglyceride with a fatty acid chain at position 2, then this lipase is 1,3 specific (Goh *et al.*, 1993). The *Humicola* lipase-catalyzed reactions gave results that are indicative of 1,3-specificity. The enzyme is also specific for chain length. *Humicola* lipase has a greater affinity for C_{16}, as compared to C_{18}, fatty acids as the activity was shown to be higher for C_{16} than C_{18} , fatty acids (Koh *et al.*, 1994).

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I would like to address another example of immobilized lipase by adsorption. Lipozyme is a fungal *Mucor miehei* lipase immobilized on a macroporus anion exchange resin. The macroporus lipozyme can be optimally used at 60-70°C. The solvent-free enzymatic interesterification reaction of palm olein which is a major fraction of palm oil, with stearic acid, was carried out in a bioreactor for 20 hr in the presence of 10% lipozyme and 1% water. The fatty acids have been interesterified with stearic acid and a new fat called cocoa-butter like fat is formed. A combination of fractionation steps gave a fat, whose triglyceride composition and melting profile were comparable to that of cocoa butter. From the HPLC analytical data, the triglyceride composition of cocoa butter-like fat is similar to that of cocoa butter. This is also true for melting profile by using differential scanning calorimeter analysis (Chong *et al.*, 1992). By using the lipozyme-catalyzed reaction, we have developed a simple one-step method to obtain palm esters and amides from various palm oil fractions or palm fatty acid distillates. These palm esters and amides are important constituents of cosmetic and pharmaceutical products. At the same time, minor components such as carotenes and vitamin E, are recovered (Goh *et al.*, 1996).

Covalently-bound Enzyme

Another group of immobilized enzyme which I wish to highlight is the covalently-bound enzyme. The porous membrane contains epoxy groups which are prepared by UV-initiated photo-polymerization of HEMA (hydroxyethylmethacrylate) and GMA (glycidyl methacrylate). The enzyme (α-amylase) is immobilized onto the poly (HEMA-GMA) membranes by means of the amide linkage formation between the amino group of the enzyme and the epoxy group of the support. The covalently-bound enzyme shows higher temperature or thermal stability (Bayramoğlu *et al.*,2004).

I am now showing you the technological procedure of micro-encapsulation, that is, the preparation of alginate-chitosan-enzyme microcapsules. Encapsulation is carried out by using two types of polysaccharides. By varying the counter ions, liquid or solid core microcapsules can be produced. By using calcium ion, liquid core is formed. On the other hand, by using barium ion as the counter ions, solid core microcapsules are formed (Taqieddin and Amiji, 2003).

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Immobilized Cells: Biotechnology and Application

I shall now turn to the biotechnology and application of immobilized cells. The application of immobilized cell biocatalysis is important in food and pharmaceutical industries. The salient feature of immobilized cell systems is the use of some confining or binding structure to constrain the cells in a particular region of the bioreactor. One main reasons of cell immobilization is to attain higher cell densities than in suspended free cell systems. Cell immobilization offers continuous separation of product and removal of reaction inhibitors. The immobilized cell containing one or more enzymes may essentially serve as immobilized enzyme biocatalyst, with the entire cell used in order to minimize treatment and processing costs.

The production of low lactose milk is a good example to illustrate the application of these immobilized cells. The occurrence of lactose intolerance resulting from the inability to utilize milk lactose is widespread. The low intestinal lactase activity may result in serious gastro-intestinal symptom. Consequently, there has been great considerable interest in the enzymatic hydrolysis of milk by lactase. However, the cost of lactase may be 20-30% of total production cost of low lactose milk. The cost of extraction of lactase is high. Furthermore, the soluble form of lactase may not be stable. In order to save cost and for continuously reuse of enzyme biocatalysts, whole cells are immobilized. The *Kluyveromyces* yeast cells with high lactase activity were first permeabilized with 10% ethyl acetate and 30% ethanol so that cells were not viable. The permeabilized cells contained only lactase were entrapped in calcium alginate cylinders by extruding the slurry of alginate-cells mixture into 25 mM calcium chloride solution. The batch conversion of lactose to glucose in 2% skim milk was 35%. In the continuous system with a dilution rate of 0.16 hr^{-1}, the percentage of bioconversion was 21.6% (Fatimah and Wang, 1991).

Another application of immobilized cells which I wish to address is the production of fuel alcohol from cellulose biomass. With the realization of the need to conserve petroleum-based fuel, interest is growing in the production of fuel alcohol from renewable ligno-cellulose biomass. Yeast cells only contain enzymes that yield alcohol from glucose. In order to utilize cellulose hydrolysate, yeast cells must co-immobilized with enzyme cellobiase. By immobilizing yeast cells and co-entrapped with cellobiase-sepharose complex in alginate cylinders, we have reported 80% yield of ethanol from cellobiose solution (Wang *et al.*, 1985).

Microbial indicators in water sources

I am now addressing the relationship between microbial indicators and water quality. The primary cause of the water-borne infection is faecal pollution. Faecal pollution refers to the access of mammalian, and especially human faeces to the water. It also means that, if the people who pollute the water are suffering from intestinal infections, those who drink the water will ingest the organisms and may be infected. The concern for municipal water supplies in temperate and tropical countries is that faecal pollution may allow the organisms which cause enteritis diseases, to be spread through the water supply and may cause a large outbreak of enteric diseases among the community. In the tropics, about 5-10% of deaths are due to water-related diseases, particularly among many young children where malnutrition and infection are the two main contributing factors.

The criteria of faecal microbial indicators had been enumerated in details by Bonde (1977). The bacteria which have gained some acceptance as indicators are the coliform, faecal coliform, *Escherichia coli* and faecal *streptococcus* (particularly the *enterococccus* group).

Bacteriophages whose hosts are strains of *E. coli* are coliphages (Bradley, 1967). They have been found to be ubiquitous inhabitants of the intestinal tract of man and animals and are encountered wherever faecal contamination occurs (D'Herelle, 1926). Guélin (1948) was the first to mention the potential of bacteriophage to act as an indicator system. Since then, numerous reports have indicated the potential of bacteriophage/coliphage to act as indicators of microbial water quality (Kennedy *et al.*, 1985; Borrego *et al.*, 1987)

Microbial Water Quality Monitoring in South-East Asia

The drinking water supplies in rural areas in Southeast Asia are often drawn from rivers and small water catchments, such as wells, holding tanks and ponds. In order to maintain water quality standards in rural communities, it is necessary to have a simple, rapid, cheap and reliable technique for monitoring the microbiological quality of such drinking water. Conventional tests for assessing water quality have several disadvantages for routine use in developing countries. They are not easily portable for

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use in rural areas and they require either trained technicians, sophisticated laboratory equipment and expensive supplies. The APHA coliphage detection method (APHA, 1985) was proposed to be one of the best methods for monitoring microbial water quality in rural areas of Southeast Asia (Sim *et al.*, 1988). The conclusion was drawn from the analysis of over 1,000 water samples from Malaysia, Thailand and Singapore by the coliphage detection method as compared with other conventional microbial water quality tests, such as most probable number and membrane filtration method.

We have also reported a significant correlation between coliphage enumerated by the APHA standard method for coliphage detection and coliforms enumerated by conventional techniques, such as the multiple tube MPN (most probable number) , A-1 MPN and m-FC (faecal coliforms) methods. Linear regression analysis of their $\log_{10}$ transformed data showed significant correlation between coliforms and coliphages for surface water ($p<0.0001$) and well water ($p<0.001$) (Loh *et al.*, 1988). We have reported the use of coliphage field kit in Malaysian rain forest (Loh *et al.*, 1990).

The coliphage field kit has been upgraded to coliphage field kit-MK I and is durable, friendly-user and smaller in size. The quality control test on nutrient medium was also carried out using Langat-1 phage particles (Dan *et al.*, 1996). A classification system based on coliphage counts was also proposed (Wang, 1995). Various classes of water from rivers of the tropical pristine forests, water intake points of treatment plants as well as raw water intake point for rural water supply in estates have been designated based on coliphage counts. These classes of water are comparable to the classes of the interim national microbiological water quality standards of Malaysia based on coliform and faecal coliform counts.

Future Direction and Initiatives

Although the coliphage field kit-MKI was found to be durable, portable and easy to use in the field and laboratories with small working spaces, it was noted that the steps for enumerating phages may still be lengthy, despite overall simplification. It was recommended that a simple qualitative assessment test for coliphage enumeration, such as dip-stick test, be developed and incorporated into the field kit (Lee, 1996). Assays for the enzyme glutamate decarboxylase (GAD) [EC 4.4.1.15] have been previously

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reported to be very selective for *E. coli*, with specificity rates, ranging from 95-97% (Rice *et al.*, 1993). We have also reported that GAD is very selective for *E. coli* isolated from the tropical waters. The high selectivity of GAD for *E. coli* may be a good and rapid assay procedure for detection of this indicator organism (Liew and Wang, 1999). The change of colour from yellow to blue is distinct and is the basis of enzyme assay for GAD. By thorough and careful design, a microbial indicator field test kit based on colour changes by GAD enzyme should be materialized soon in coming years.

With these future research initiatives floating in your mind, I would like to thank all of you for you kind attention. Thank you very much.

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I wish to thank the University of Malaya for providing me with excellent research facilities and atmosphere to carry out my research. I also wish to thank my wife and two children for their constant supports and encouragement.

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