ISOLATION OF ALKALOPHILIC MICROORGANISM FROM LOCAL HOT SPRING

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DECLARATION

I declare that this thesis entitled “Isolation of Alkalophilic Microorganism from Local Hot Spring” is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.”

Signature :……………………………………….

Name of Candidate : Mohd Zulhilmi bin Wan Salleh

Date : May, 2008

DEDICATION

Special Dedication to my Mother and Father,

My family members that always love me,

My friends, my fellow colleague,

and all faculty members.

For all your Care, Support and Believe in me.

Sincerely

Mohd Zulhilmi bin Wan Salleh

ACKNOWLEDGEMENTS

Bismillahirrahmanirahim,

I am so thankful to Allah S.W.T for giving me patient and spirit throughout this project and the research is successfully complete. With the mercifulness from Allah therefore I can produces a lot of useful idea to this project.

To my beloved father and mother, Wan Salleh bin Ismail and Saodah binti Abdullah .I am grateful to have both of you in my life and giving me full of support to through this life. I pray and wish to both of you are always in a good health and in Allah mercy. You are the precious gift from Allah to me.

I am indebted to my supervisor, Mr. Rozaimi bin Abu Samah, lecturer from the Faculty of Chemical Engineering and Natural Resources for his advice, insightful comments and support. Thank for your guide and without your guide this research will not complete and well organized.

I would like to dedicate my appreciation to all the lecturers that involve in this project for their invaluable time, guidance and advice. I would also like to thank all my beloved friends especially from first batch biotechnology students who have accompanied me throughout this project. Thank you very much

ABSTRACT

Alkaline environments are typical extreme environments which include naturally occurring soda lakes, deserts, soils and artificially occurring industrial-derived waters. Micro-organisms that occupy extreme pH environments have resulted in the definition of an unusual group, termed alkaliphiles. In this review, the current status of the biodiversity of alkaliphilic micro-organisms in hotspring environments and aspects of their biotechnological potential are summarized briefly. The sample was taken from the local hot spring which is Sungai Klah, Perak and the temperature at its condition was recorded. The sample was inoculated in alkaline medium or known as Hirokoshi medium and incubated for the growth of the culture. By using enrichment technique, sample from each culture were spread on selective agar plate. As a result, only alkalophilic microbes grow by using selective agar plate. Alkalophilic microorganism was characterized. In this experiment, morphology test has been done to determine its shape, size and arrangement. The microbe was characterized using simple staining, gram staining, spore staining and acid-fast staining. The results gave a microorganism with rod in shape, gram negative microbes, spore former and also non acid-fast cells Therefore, according to the morphology test, it was best described the alkalophilic microorganism that had been study is from *Bacillus* group.

ABSTRAK

Persekitaran beralkali adalah kawasan yang ekstrim termasuk tasik soda semulajadi, gurun, tanah, dan sisa air dari industri. Mikroorganisma yang menetap di kawasan yang mempunyai nilai pH tinggi ini adalah terdiri dari kumpulan yang jarang dijumpai dinamakan 'alkaliphiles'. Dalam tesis ini, diterangkan secara ringkas pengenalan dan keadaan kepelbagaian biologi iaitu mikroorganisma yang beralkali dan aplikasinya. Sampel air di ambil dari kolam air panas tempatan iaitu Sungai Klah, Perak dan suhu tempat sampel diambil direkodkan. Kemudiannya, sampel dihidupkan di dalam media yang beralkali ataupun di kenali sebagai Hirokoshi media dan di biakkan di dalam inkubator. Dengan menggunakan teknik pengkayaan, sampel dari setiap kultur diratakan di atas media terpilih. Mikroorganisma ini akan dibuat pengelasan ciri untuk menentukan bentuk dan ciri yang dibawa. Beberapa ujian dijalankan iaitu `*simple staining', `gram staining',`spore staining'* dan `*acid fast staining'*. Hasilnya, mikroorganisma ini mempunyai bentuk rod, mikroorganisma `*gram negative'* ,kumpulan spora dan bukan daripada kumpulan `*mycobacterium'*. Berdasarkan ujian morfologi yang dijalankan, mikroorganisma beralkali yang memenuhi semua ciri-ciri ini adalah berkemungkinan dari kumpulan *Bacillus*.

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LIST OF SYMBOLS

%	-	percent
°C	-	°Celcius
µg/ml	-	microgram per mililiter
g	-	gram
g/ml	-	gram per mililiter
kg	-	kilogram
L	-	liter
L/h	-	liter per hour
ml	-	mililiter
mm	-	milimeter
nm	-	nanometer
wt/vol	-	weight per volume
wt %	-	weight percent

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

The discovery of alkaliphiles was fairly recent. Only 16 scientific papers on the topic could be found when Koki Horikoshi who is the professor from Department of Bioengineering Tokyo Institute Technology Tokyo, Japan started experiments on alkaliphilic bacteria in 1968. The use of alkaliphilic microorganisms has a long history in Japan, since from ancient times indigo has been naturally reduced under alkaline conditions in the presence of sodium carbonate. Indigo from indigo leaves is reduced by particular bacteria that grow under these highly alkaline conditions in a traditional process called indigo fermentation. The most important factor in this process is the control of the pH value.

Organisms which thrive in extreme alkaline environments offer us the opportunity to appreciate the range of adaptive possibilities that evolution can bring to bear on fundamental biological processes and they constitute unique models for investigations on how biomolecules are stabilized when subjected to extreme conditions. Alkaliphilic microorganisms offer a multitude of actual or potential applications in various fields of biotechnology. Not only do many of them produce compounds of industrial interest, but they also possess useful physiological properties which can facilitate their exploitation for commercial purposes. The bioenergetics that alkaliphiles face in maintaining pH homeostasis in a highly alkaline environment has been increasingly studied in the last two decades.

1.2 Problem Statement

It is well known that 'moderate' environments are important to sustain life. 'Moderate' means approximately neutral pH, temperatures between 20^{o}C and 40^{o}C, air pressure about 1 atm and adequate concentration of nutrient and salts. However, in recent times, many organisms have been found in such extreme environments and can produce enzymes that are active and stable at high temperature and pH values. Thus, this idea of extreme environments is so benefit and we must extend our consideration to other environments in order to isolate and cultivate new microorganism

1.3 Objective

To isolate microorganism from local hot spring in alkaline environments.

1.4 Scopes of Studies

The scopes of this study were as follows:

i) To isolate the alkalophilic microorganism
ii) To screen the alkalophilic microorganism
iii) To characterize the morphology of the alkalophilic microorganism.

CHAPTER 2

LITERATURE REVIEW

2.1 Thermophiles

It is believed that extreme environmental conditions existed on the surface of the earth before they were modified by the organisms through metabolism, and from cooling of the crust (Schwartzman, 1997) Therefore, research on extremophiles provides clues for deciphering the origin and sustenance of life on prehistoric earth. Furthermore, the study of microbes that are known to thrive under extreme salinity, acidity and temperature conditions will not only help us understand how life originated and diversified on earth but also how life may thrive on other planets.

Thermophiles, a class of extremophile that has a temperature optimum for growth at around 45°C or higher is of particular interest (Stetter, 1996). Within the domains Archaea and Bacteria, thermophiles are among the most deeply branching organisms on the phylogenetic tree, based on 16 S rRNA sequences (Pace, 1997) Thus they are the most primitive group of organisms known on this planet and possibly are closely similar to the common ancestor (Canfield and Raiswell 1999). They were also lowest to evolve and may represent ancient adaptations to heat. Furthermore, it has been proposed that hydrothermal systems with characteristically high temperatures may have provided favourable environments for the prebiotic synthesis of organic compounds necessary for life and may also have been a site for life's origin (Shock, *et al.,* 1998). In order for the nascent life to sustain and proliferate, metabolic energy is essential.

Thermophilic micro-organisms are of special interest to enzymologists both at the fundamental and industrial level as a natural source of enzymes that are active and stable at high temperatures.

Moreover, the interest in enzymes with adequate thermostability is of importance for industrial applications such as in food and feed, paper and pulp, modification of complex polysaccharides and in organic biosynthesis because they are stable in presence of denaturing agents and organic solvents.

2.2 Alkaline Environments

Naturally, the existence of alkaline environments because of the presence such as carbonate springs, alkaline soils, and lakes. The alkaline condition is characterized by their highly basic pH values ranging from 8 to 11. Soda lakes, which represent stable and extremely productive aquatic ecosystems, exhibit ambient pH values around 10 or higher. Naturally occurring alkaline environments found worldwide (Grant and Tindall 1986).

These environments are characterized by high concentrations of Na_2CO_3 (usually as $Na_2CO_3.10H_2O$ or $Na_2CO_3.NaHCO_3.2H_2O$). The distinguishing feature of soda lakes is depleted Mg^{2+} and the presence of this carbonate provides buffering capacity to the lake waters (Grant *et al.,* 1990). The formation of alkalinity in the soda lake environment requires a combination of geographical, topographical and climatic conditions: firstly, the presence of geological conditions which favor the formation of alkaline drainage waters; secondly, suitable topography which restricts surface outflow from the drainage basin; and thirdly, climatic conditions conducive to evaporative concentration. Such conditions are found in arid and semi-arid zones of tropical or subtropical areas.

A vital condition necessary for the formation of a soda lake is that significant amounts of Ca^{2+} and Mg^{2+} must be absent so that ground waters containing HCO_3^-

are produced where the molar concentrations of HCO_3^- / CO_3^- greatly exceed those of Ca^{2+} and Mg^{2+}. Through evaporative concentration, such waters rapidly achieve saturation with respect to alkaline earth cations which precipitate as insoluble carbonates, leaving Na^+, Cl^- and HCO_3^- / CO_3^- as the major ions in solution (Grant and Horikoshi, 1989) Alkalinity develops due to a shift in the CO_2 / HCO_3^- / CO_3^{2-} equilibrium as: $2HCO_3CO^2 + CO^{2-} + 2H^2O$. Alkalinity evolves concomitant with the precipitation of other ions, especially Na^+ and Cl^-, leading to the development of alkaline and saline conditions. The relative salinity of any lake is dependent on the local geologic and climatic conditions, resulting in saline, alkaline lakes. In lakes of lower salinity, the concentration of CO_3^{2-} usually exceeds that of Cl^-, but in brines of higher salinity Cl^- exceeds CO_3^{2-} concentrations (Jones *et al.,* 1994)

2.3 Alkaliphiles

There are no precise definitions of what characterizes an alkaliphilic or alkalitolerant organism. Several microorganisms exhibit more than one pH optimum for growth depending on the growth conditions, particularly nutrients, metal ions, and temperature. Therefore, the term "alkaliphile" is used for microorganisms that grow optimally or very well at pH values above 9, often between 10 and 12, but cannot grow or grow only slowly at the near-neutral pH value of 6.5 (Horikoshi, 1999a) In this review, therefore, the term “alkaliphile” is used for microorganisms that grow optimally or very well at pH values above 9, often between 10 and 12, but cannot grow or grow only slowly at the near-neutral pH value of 6.5 as shown in Figure 2.1.

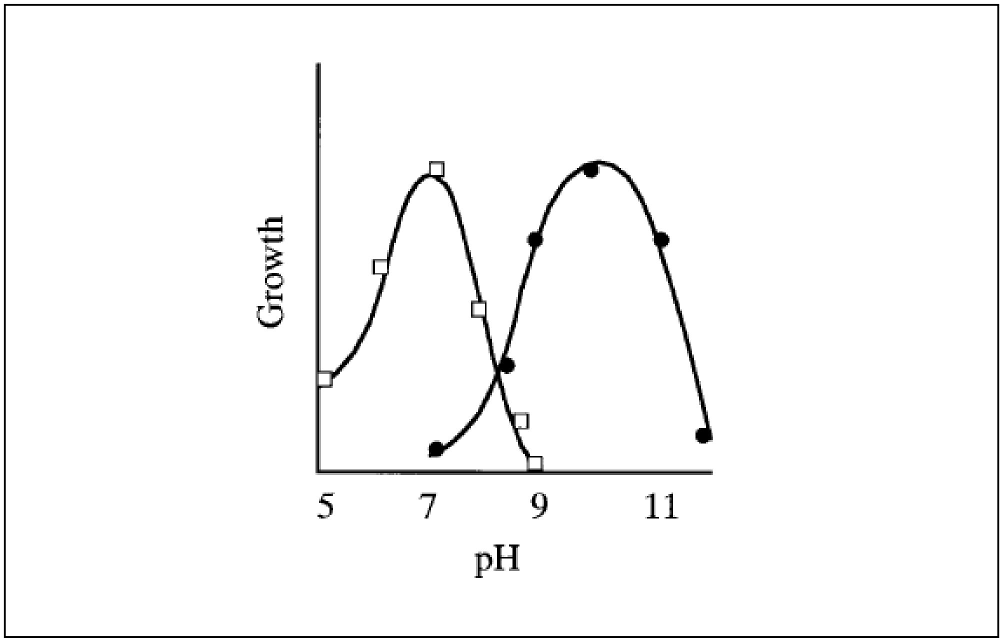

Figure 2.1: The pH dependency of alkaliphilic microorganisms. The typical pH dependency of the growth of neutrophilic and alkaliphilic bacteria is shown by open squares and solid circles, respectively (Horikoshi, 1999b).

2.3.1 Distribution Of Alkaliphiles

Alkaliphiles consist of two main physiological groups of microorganisms; alkaliphiles and haloalkaliphiles. Alkaliphiles require an alkaline pH of 9 or more for their growth and have an optimal growth pH of around 10, whereas haloalkaliphiles require both an alkaline pH 9 and high salinity (up to 33% (wt/vol) NaCl) (Horikoshi, 1999c). Alkaliphiles have been isolated mainly from neutral environments, such as soil as shown in Figure 2.2 and sometimes even from acidic soil samples an. Haloalkaliphiles have been mainly found in extremely alkaline saline environments, such as the Rift Valley lakes of East Africa and the western soda lakes of the United States.

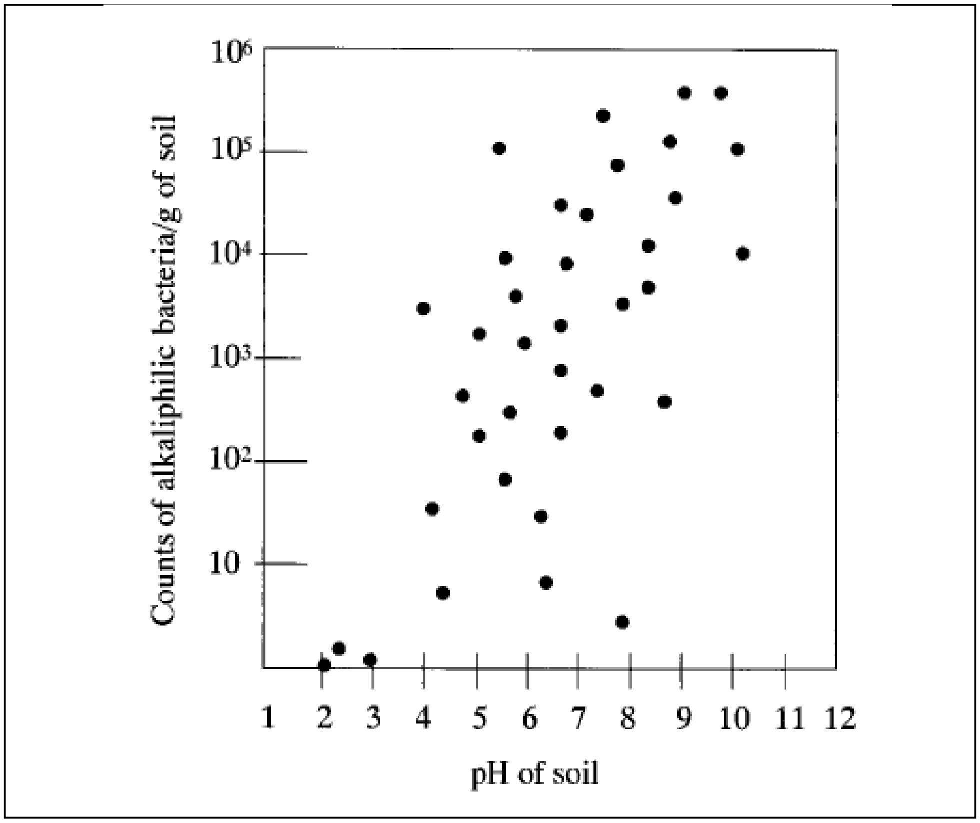

Figure 2.2: Distribution of alkaliphilic microorganisms in environments at various pHs. (Horikoshi, 1999a).

2.3.2 Types of Alkaliphiles

Alkaline-adapted micro-organisms can be classified into two main groupings, alkaliphiles (also called alkalophiles) and alkalitolerants. The term alkaliphiles (alcali from Arabic, soda ash, phile, loving) is generally restricted to those micro-organisms that actually require alkaline media for growth. Table 2.1 shows basal media for the growth of alkaliphilic microorganism. The optimum growth rate of these icro-organisms is observed in at least two pH units above neutrality. Organisms capable of growing at pH values more than 9 or 10, but with optimum growth rates at around neutrality or less, are referred to as alkalitolerant (Jones *et al.,* 1994; Grant *at al.,* 1980). Table 2.2 shows the summary of the alkaliphilic microorganism by its types.

Table 2.1: The basal media for alkaliphilic microorganisms (Horikoshi, 1999a).

Ingredient	g/L
Soluble starch	10
Polypeptone	5
Yeast extract	5
KH2PO4	1
Mg2SO4. 7H2O	0.2
Na2 CO3	10
Agar	20

Table 2.2: The alkaliphilic microorganism by its types.

Types	Name of microorganism	References
Aerobic alkaliphiles	*Bacillus* *Micrococcus* *Pseudomonas* *Streptomyces*	Kimura *et al.,* 1987
Anaerobic	*Clostridium thermohydrosulfuricum* *Clostridium paradoxum* *Amphibacillus xylanus.* *Thermococcus alcaliphilus*	Podkovyrov and Zeikus (1992) Cook *et al.,* 1996 Kodama and Koyama (1997) Tanabe *et al.,* 1988
Haloalkaliphiles	*Natronobacterium magadii* *Natronobacterium Pharaonis* *Natronomonas Pharaonis* *Natronobacterium Vacaolatum* *Halorubrum vacuolatum* *Natrialba magadii.* *Halobacterium Trapanicum* *Natrialba asiatica* *Tindallia magadii*	Lodwick *et al.,* 1994 Kamekura *et al.,* 1997 Xu *et al.,* 1999 Kevbrin *et al.,* 1997
Cyanobacteria	*Synechocystis* *Nostoc calcicola*	Buck and Smith (1995)

2.4 Alkaline enzymes

The advances in the application of alkaliphilic or alkalitolerant-based biomolecules during the past 20 years are due in the main to the introduction of proteolytic enzymes classified as serine protease in the detergent industry. Since the discovery of this enzyme in the 1970s, attention has been centred on alkaliphilic enzymes so that within a few years a large number of enzymes became available.

Industrial applications of alkaliphiles have been investigated and some enzymes have been commercialised. Of the enzymes now available to industry, enzymes such as proteases, cellulases, lipases, pullulanases are by far the most widely employed and they still remain the target biomolecules. Due to the unusual properties of these enzymes they are expected to fill the gap between biological and chemical processes and have been greatly employed in laundry detergents. Table 2.3 shows some present enzymes produce by alkalophilic microorganism.

Table 2.3: Enzymes produced by alkalophilic microorganism.(Horikoshi, 1999a)

Microorganism	**Enzyme**
Staphilothermus marinus	Amylase
Bacillus thermoleovocas	Lipase
Thermococcus litoralis	Pullulanase
Clostriridium absonum	Cellulase
Bacillus circulans	Xylanase
Fusarium proliferation	a-Galactosidase
Clostridium abosum	Penicillinase
Pyrococcus abyssi	Phosphatase

2.5 Application of alkalophilic microorganism

Organisms which thrive in extreme alkaline environments offer us the opportunity to appreciate the range of adaptive possibilities that evolution can bring to bear on fundamental biological processes and they constitute unique models for investigations on how biomolecules are stabilized when subjected to extreme conditions. Alkaliphilic micro-organisms offer a multitude of actual or potential applications in various fields of biotechnology. Not only do many of them produce compounds of industrial interest, but they also possess useful physiological properties which can facilitate their exploitation for commercial purposes.

2.5.1 Ability to Degradation of Macromolecules

The ability of alkalophilic microorganism to degrade macromolecules efficiently have attractive the researcher to study its potential deeply. Alkaline environments are colonized by a variety of bacterial populations which might play a role in the chemical breakdown of certain macromolecules. Few studies have treated alkaliphilic species, and especially their degradation of phenolic-lignin compounds (Horikoshi *et al.,* 1999). These species so far appear to degrade lignin. Lignin degradation of alkaliphilic bacteria has been studied very little. More extensive