ISOLATION AND SCREENING OF LIPASE, PROTEASE, CELLULASE AND AMYLASE PRODUCING BACTERIA FROM OIL CONTAMINATED SOIL

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Abstract - Some industries or processes such as waste water treatment process, textile industry and detergent making process require multiple enzymes. Among microbes, Bacilli species can produce multiple extracellular enzymes. In this experiment, spore forming bacteria were isolated from oil contaminated soiland theirextracellular enzyme production and enzymatic activity indexes were screened at pH 7 and 37℃ and calculateded. Different pH and temperature were examined for best growth. Morphological characteristic and biochemical tests revealed that all were gram positive. Among seven isolated strains, six strains were aerobic, rod-shaped Bacillus species and the left one which produced only two kinds of enzymes was facultative anaerobic, filamentous Streptomyces. Out of seven isolated strains, three strains (S1, S3 and S7) secreted lipase, protease, cellulase and amylase, three others (P1, P2 and P11) secreted lipase, protease and cellulase and the left (P105) lipase and protease only.

Keywords - Bacilli Species, Multi Extracellular Enzyme Producing Bacteria, Oil Contaminated Soil, Spore Forming Bacteria

I. INTRODUCTION

Living microbes decompose or break down organic substances or artificial materials that are similar enough to plant and animal matter into smaller compounds through metabolic or enzymatic processes [1], [5]. This process is known as biodegradation. Biodegradation process can be aerobic, with oxygen, or anaerobic, without oxygen. Several microorganisms, including fungi, bacteria and yeasts are involved in biodegradation process. Algae and protozoa reports are scanty regarding their involvement in biodegradation [1], [6].

The term biodegradation is often used in relation to ecology, waste management and mostly associated with environmental remediation (bioremediation) [1], [2]. In bioremediation process, there are three phases or levels. First, through natural attenuation, contaminants are reduced by native microorganisms without any human augmentation. Second, biostimulation is employed where nutrients and oxygen are applied to the systems to improve their effectiveness and to accelerate biodegradation. Finally, during bioaugmentation, microorganisms are added to the systems. These supplemental organisms should be more efficient than native flora to degrade the target contaminant [1], [7]. A feasible remedial technology requires microorganisms being capable of quick adaptation and efficient uses of pollutants of interest in a particular case in a reasonable period of time [1], [8].

The decomposition process of the organic material consists of various types of heterotrophic bacteria such as proteolytic, cellulolytic, amylolytic, nitrification and denitrification [3], [4]. Heterotrophic bacteria obtain energy from oxidation of organic compounds. Carbohydrates (particularly glucose), lipids, and protein are the most commonly oxidized compounds. Biologic oxidation of these organic compounds by bacteria results in synthesis of ATP as the chemical energy source. This process also permits generation of simpler organic compounds (precursor molecules) needed by the bacteria cell for biosynthetic or assimilatory reactions. Another type of heterotrophic metabolism is fermentation in which an organic compound rather than oxygen is the terminal electron (or hydrogen) acceptor. Less energy is generated from this incomplete form of glucose oxidation, but the process supports anaerobic growth [9].

Kitchen waste and waste water consist of different kinds of oxidized compounds. In the treatment of these wastes as bioaugmentation, if addition of a single species with certain requirements for industrial production can produce multiple extracellular enzymes, it is feasible to manipulate the process [10].

Apart from this advantage, multiple extracellular enzymes are used rather than single enzyme in textile industry and detergent making process.

Accordingly, the purpose of this experiment wasto study the growth of the isolated strains at different temperatures and pH,to screen the enzymes the isolated strains secreted and to calculate the enzymatic activity indexes of those enzymes.

II. MATERIALS AND METHODS

A. Materials

All the chemicals in this study were of analytical grade and bacteriological grade. All of them were purchased from HiMedia, USA and Kermel Chemical Reagent Co., Ltd., China. For lipase screening, olive oil imported from Spain was commercially available in local market. The other essential instruments such as ALP autoclave (CLG-32LDVP), ESCO streamline class II biological safety cabinet, POL-EKO laboratory incubator and Bioblue lab binocular microscope were used for this experiment.

B. Culture Media for the Strain

In this experiment, three different culture media were used. To isolate spore forming bacteria, bacterial grade nutrient agar was used. To maintain and study their enzymatic activity, 10 g/L starch, 5 g/L peptone, 5 g/L yeast extract, 15 g/L agar, pH 7.0 was used for Bacillus sp. and 10 g/L starch, 0.3 g/L casein, 2 g/L KNO_3, 2 g/L NaCl, 2 g/L K_2HPO_4, 0.05 g/L $MgSO_4$, 0.02 g/L $CaCO_3$, 0.01 g/L $FeSO_4$, 15 g/L agar, pH 7.0 for Streptomyces sp..

C. Sample Collection

Oil contaminated soil samples were collected from an edible oil processing mill in Sagaing Region and a garage in Mandalay Region. Sample collection was carried out by using sterile spatulas and sterile polyethylene bags. Before being used, each sample was stored under lab condition.

D. Isolation of Spore Forming Bacteria

One gram of each sample was mixed with 10 ml of sterilized warm nutrient broth (80℃). The mixtures were stirred thoroughly until 37℃ and the suspension of two different soil samples was placed on two nutrient agar plates through streaking method [11], [12]. The plates were incubated at 37℃ for 48 hours until colonies appeared. Morphologically distinct colonies were picked up and inoculated on the nutrient agar plates at 37℃ for 24 hours.

E. Morphological Characterization

After sub-culture had been done for 6 times, the pure strains were obtained. To distinguish the Gram-positive or Gram-negative bacteria,Gram staining and microscopic viewings were carried on. The cultures on different selected media were also incubated at 37℃ for 24 hours.

F. Biochemical Characterization

Catalase test, Oxidase test, Citrate utilization test, Methyl-red test, Voges–Proskauer test and Gelatin liquefaction test were performed to identify the isolated bacteria.

G. Determination of Enzymatic Activity

Enzymatic activities such as lipolytic, cellulolytic, proteolytic and amylolytic activities of the isolated bacteria were studied by inoculating them onto the respective agar mediacontaing substrate that can show specific enzymatic activity.The enzymatic activity indexes for these enzymes were expressed by the ratio of the average diameter of the degradation halo to the average diameter of the colony growth [13], [14].

H. Screening for Lipase Activity

The isolates were inoculated in the 1% (v/v) Tributyrin agar plates and incubated at 37℃ for two days. In this test, 1% (v/v) Tributyrin was added to respective culture media. Clear zones around the culture colonies indicated lipase activity [15].

I. Screening for Protease Activity

The isolates were inoculated onto the respective culture media agar enriched with 1% skim milk and incubated at 37℃ for two days. Clear zones around the culture colonies indicated protease activity [13], [14].

J. Screening for Cellulase Activity

Cellulase activity was screened by inoculating the isolates in the respective culture media agar enriched with 1% CMC and incubated at 37℃ for two days. Clear zones around the culture colonies were visualized by flooding the culture plates with 0.1% Congo red for a few minutes and washing them with 1M NaCl [14], [16].

K. Screening for AmylaseActivity

The strains were inoculated onto the respective culture media agar containing 1% (w/v) starch. After incubation for two days at 37°C, agar plate surfaces were treated with iodine solution, which allowed to observe unstained zone around active amylase colonies [14], [17].

III. RESULTS AND DISCUSSION

A. Results

From the oil contaminated soil samples, seven endospore forming bacteria were able to be isolated. According to Gram staining and biological characterization, all of the strains were identified as Gram-positive. Among them, six of the strains were Bacillus species and the left one was Streptomyces species according to conventional analysis as detailed in Table I.

Isolated strains P1, P2 and P11 could grow well in both of the selected media used in this study but S1, S3, S7 and P105 did not. The growth of S1, S3 and S7 required a little more yeast extract than P1, P2 and

P11 according to this study. P105 grew well in the mineral rich medium used in this study.The effect of pH on the bacterial growth was studied by adjusting the pH of culture medium with 0.1N NaOH and 0.1N HCl to pH 5, 7 and 9. This pH effect study was carried out at 37℃. The result revealed that the isolated strains P1, P2, P11 and S1 were resistant to pH changes and able to grow at pH 5. Among the others, S3 and S7 were a little more resistant to pH changes than P105 but these three strains preferred to grow at high pH.The study of the effect of temperature on their growthat 17 ℃, 25 ℃ and 40℃was investigated at pH 7.In this investigation, P105 grew well in the range of temperature 17℃ to 40℃ but the growth of P1, P2, P11, S1, S3 and S7 was favoured at high temperature. The detailed results were shown in Table I. In the observation of enzymatic activity, S1, S3 and S7 showed lipolytic, cellulolytic, proteolytic and amylolytic activities, P1, P2 and P11 showed lipolytic cellulolytic and proteolytic activities and P105, only lipolytic and proteolytic activities as detailed in Fig 2.

To calculate index of enzymatic activity, all experiments were performed in duplicate. The values of measurement were taken in average. From the calculation, proteolytic indexes of S1, S7 and P105 were 1.97, 1.96 and 1.87 respectively. The cellulolytic and amylolytic indexes of S3 were 1.8 and 1.08 and the lipolytic index of P1, 0.89.

Test items	Isolated strains						
	P1	P2	P11	S1	S3	S7	P105
Gram staining	+	+	+	+	+	+	+
Cell shape	small rod	small rod	small rod	large rod	large rod	small rod	filamentous
Catalase	+	+	+	+	+	+	-
Oxidase	+	+	+	+	+	+	-
Voges-Proskauer test	+	+	+	+	+	+	-
Methyl red test	-	-	-	+	+	+	-
Citrate utilization test	+	+	+	+	+	+	+
Indole test	+	+	+	+	+	+	-
Growth at pH 5	++	++	++	++	+	+	-
Growth at pH 7	+++	+++	+++	+++	+++	+++	+++
Growth at pH 9	+++	+++	++	+++	+++	+++	+++
Growth at 17℃	++	++	++	++	++	++	+++
Growth at 25℃	+++	+++	+++	+++	+++	+++	+++
Growth at 37℃	+++	+++	+++	+++	+++	+++	+++
Growth at 40℃	+++	+++	+++	+++	+++	+++	+++

Table I. The physiological characteristics of isolated strains

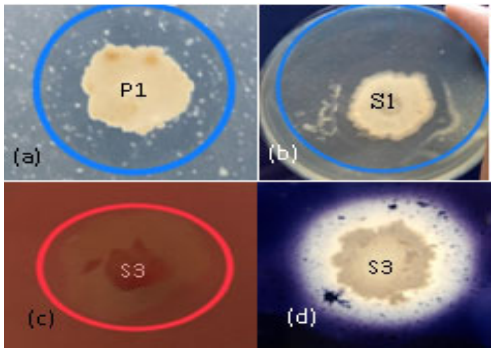


Fig. 1. The clear zone of enzymatic activity (a) lipase, (b)protease, (c) cellulase, (d) amylase

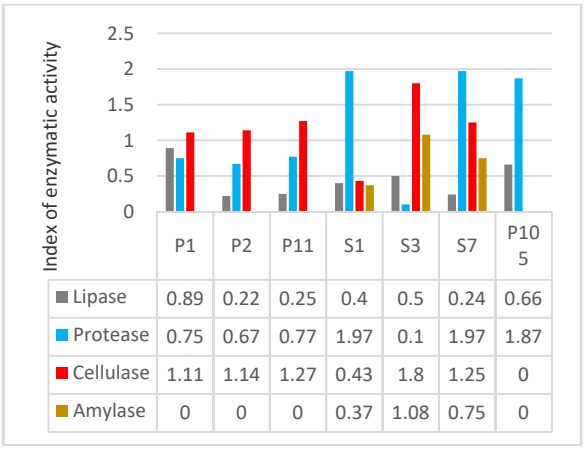


Fig. 2. Chart of index of lipolytic, proteolytic, cellulolytic and amylolytic activities

B. Discussion

In this study, edible oil and petroleum hydrocarbon contaminated soils were used for isolation of spore forming bacteria which produced multiple extracellular enzymes. Heat shock allowed the selection of microorganisms in spore form [13]. By using warm sterilized nutrient broth (about 80℃), four strains (P1, 2, 11 and 105) were isolated from petroleum hydrocarbon contaminated soil and three strains (S1, 3 and 7) from edible oil contaminated soil.

Microbes have more adaptation abilities and inhospitable atmospheres like Dead Sea, Antarctica, Alkaline lakes, Hot springs, volcanic vents and contaminated soils, which provides extraordinary potential for the lipases production with specific features. Mostly the bacterial genera for the production of lipases and phospholipases which have been reconnoitered are Pseudomonas, Bacillus and Streptomyces [18]. Among the bacteria, the genus Bacillus has a very prominent place in terms of the commercial production of proteases. Actinomycetes, a group of bacteria having morphology similar to fungi are also known to produce proteases [19]. Anaerobic microbial species have limited cellulolytic activity [20]. Several bacteria have been shown are capable of producing a tremendous amount of alpha-amylase for industrial applications, these bacteria include Bacillus amyloliquefaciens, Bacillus licheniformis, and Bacillus stearothermophilus[21]. Many literatures have reported that Bacillus species can produce variety of enzymes [22].

Aerobic Bacillus species (P1, P2, P11, S1, S3 and S7) had lipolytic, proteolytic and cellulolytic activities. Facultative anaerobic Streptomyces species (P105) had only lipolytic and proteolytic activities. Amylolytic activity was found in three of these aerobic Bacillus species, S1, S3 and S7only.

The effects of pH andtemperature on the growth of isolated strains were studied but the experiments for

the enzymatic index calculation were done at37℃ and pH 7.These are the qualitative results of bacteria isolated from oil contaminated soil with screening for enzymatic activity on solid agar plates.

IV. CONCLUSION

From the result of this study, it can be concluded thatthere was no difference on the growth of all isolates by the effect of temperature and pH in the range of 25℃ - 40℃ and pH of 7.In addition, all the isolated spore-forming bacteria have two common hydrolytic activities (lipase and protease). Moreover all Bacillus species also showed cellulase activity. Amylase activity can be seen in only S1, S3 and S7 which were isolated from edible oil contaminated soil.On the base of this presented result,quantitative determination of enzymes from isolated strains will be done for further study and to be applied in industrial use.

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