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STUDY ALKALINE PROTEASE ACTIVITY OF *BACILLUS* SP. Z.D.H ISOLATED FROM ALKALINE PETROCHEMICAL WASTEWATER

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ABSTRACT

Alkaline protease are produced by both neutralophilic and alkaliphilic microorganisms. These two groups represent almost all sources of commercial alkaline proteases currently available in the market. After the enrichment, samples culture in Horikoshi medium supplement to skimmed milk and culture on same plate agar. Formation of halo zone around the colonies, show is proteolyses activity. The isolates which showed the largest halo zones were selected for morphological and biochemical characterization and protease activity assay. Finally confirm isolated bacteria to 16 s r RNA sequencing. The is isolated strain belonged to Bacillaceae family. Then sequenced to 16 s rRNA a new strain was identified by the name *Bacillus* sp. Z.D.H. Maximum protease production was observed at pH 9 and incubation at optimum temperature 30ºC in 24h. Alkaline petrochemical wastewater isa excellent source for isolated extremozymes producing bacteria.

Keywords: Alkaline Protease Activity, Bacillus, Alkaline Petrochemical Wastewater

INTRODUCTION

Proteases are industrially important enzymes us ed in the detergent, food, pharmaceutical, leather industries, in peptide synthesis and also have application in silver recovery from photographic plates which account for about 60% of total industrial enzyme sales **[1]**. Alkaliphiles require an alkaline pH of 9 or more for their growth and have an optimal growth pH of around 10. Many different kinds of alkaliphilic microorganisms, including bacteria belonging to the genera *Bacillus*, *Micrococcus*, *Pseudomonas*, and *Streptomyces* and eukaryotes such as yeasts and filamentous fungi, have been isolated from a variety of environments **[2]**. Alkaline protease are produced by both neutralophilic and alkaliphilic microorganisms. These two groups represent almost all sources of commercial alkaline proteases currently available in the market **[3]**. Studies of alkaliphiles have led to the discovery of many types of enzymes that exhibit interesting properties. Industrial applications: detergent additives, dehairing, other applications. Diverse industrial activities including food processing (KOH mediated removal of potato skins), cement manufacture (or casting), alkaline electroplating, leather tanning, paper and board manufacture, indigo fermentation and rayon manufacture, and herbicide manufacture generate anthropogenic sources of alkalinetype environments **[4-5]**.

Since the discovery of this enzyme in the 1970s, attention has been centered on alkaliphilic enzymes so that within a few years a large number of enzymes became available such as Alkaline Protease, Amylase, Pectinase, Pullulanase, Cellulase, Alginase, Catalase, RNase, DNase, Restriction enzyme, β1,3-Glucanase, Xylanase, a-Galactosidase, b-Galactosidase, Penicillinase, Maltose dehydrogenase, Glucose dehydrogenase, Uricase, Polyamine oxidase, b-Mannanase, and b-Mannosidase **[6]**.

MATERIAL AND METHODS

Sample Collection

In this study samples were collected from Alkaline petrochemical wastewater Asaluyeh company, Iran. The wastewater contains multiple hydrocarbons and pH of the wastewater were 12.5.

Isolation of Alkaline Protease Producing Bacteria

Isolation of alkaline protease producing bacteria was to using Horikoshi medium **[7]** containing (g/900 ml): Glucose 10, Polypeptone 5, Yeast extract 5, KH_2PO_4 1, Mg_2SO_4 $7H_2O$ 0.2, So Autoclave at 121ºC for 15 minutes. After autoclaving, aseptically add 100.0 ml of sterile 10% Na_2CO_3 to the medium.Adjust for final pH of 10.0.Agar for plats 15 g/l. and supplement skimmed milk 10.water samples inoculated in Horikoshi medium and incubated at 37ºC with aeration for 2 day to 180rpm.After the enrichment, samples culture in Horikoshi medium supplement to skimmed milk Formation of halo zone around the colonies, show is proteolyses activity. These colonies were isolated andthe isolates which showed the largest halo zones were selected forfurther studies.

Characterization of the Isolates

The characters of the isolate were studied following thestandard microbiological methods as described in Bergey's Manual of Systematic Microbiology .Gram reaction, colonymorphology. The physiological and biochemical characters, included: oxidase, catalase, indole, SH_2, MR, VP, tryptophanede aminase, gelatinase and fermentation oxidation of the following carbon sources.

Analyses of 16S rRNA Gene Sequences

For thesequence analysis, bacterial genomic DNA wasextracted and purified using phenol-chloroform method. Two primers annealing at the 5' and 3' end of the 16S rDNA were27F (5'-AGAGTTTGATCMTGGCTCAG -3') and 1492R (5'- GGTTACCTTGTTACGACTT -3') [8].PCR amplificationwas performed in a final reaction volume of 50µl,and the reaction mixture contained each primer at a volume of 1µl,in Master mix (Takara, Japan) and sample to volume 5 µl. Amplification consisted of a 1 min denaturation step at 94ºC, a minute annealing step at 58ºC and a minute extension step at 72ºC. The first cycle was preceded by incubation for 5 min at 94ºC. After 35 cycles, there was a final 10-min extension at 72ºC. Negative controls containing no DNA template were included in parallel.PCR products were separated in a 1.5% (w/v) agarose gel and were subsequently visualized by ultraviolet (UV) illumination after ethidium bromide staining.

Production of Alkaline Proteases

Protease production inoculated into 250ml Erlen containing 100ml of alkaline protease production medium, contained (g/l): Glucose 10, Peptone 5, Yeast extract 5, $KH_2PO_4$1, Mg_2SO_4 $7H_2O$ 0.2, $Na_2CO_3$10 and incubated overnight at 180 rpm and 37°C for 72 h.Cells andinsoluble materials were removed by centrifugation at 9 000 g for 30 min at 4°C and the cell-freesupernatant was used as the source of crudealkaline protease enzyme.

Alkaline Protease Assay

Alkaline protease activity was estimated by the method of Lowery **[9]**. Colonies in 100 ml protease production medium culture and incubated at 37 ° C and for 72 h .Then Solution centrifuged at 9000 rpm for 30 min and was separated the supernatant.1 ml substrate containing 1% casein in phosphate buffer (pH:7) was added to 1 ml of diluted enzyme and was incubated at 55ºC for 3 hours. In the next step stop the reaction by adding 2 ml TCA (5 %) trichloroacetic acid).Mix was incubated for 20 min. The precipitated protein was then passed through a No. 1 What man paper to 1 ml filtered material was added 0.5 ml solution of sodium bicarbonate. The final step isadd 1 ml Folin reagent and incubated for 20 min at 55 ° C until color developed the green color was measured at 660 nm wavelength.

Effect of pH and Temperature on Production Alkaline Protease

The influence of pH on production alkaline protease was determined by measuring the enzyme activity at varying pH values ranging from 5 to 11 at 30 ºC using protease production medium to different pH. The optimum temperature of the alkaline protease production was determined by incubationof the reaction mixture (pH 10.5) for 24h, at different temperatures ranging from 30 to 50 ºC (30, 37, 45, 50ºC) and measure the activity protease.

RESULTS AND DISCUSSION

Isolation Alkaline Protease Producing Bacteria

In the present study, aonebacteria to largest halo zones fromalkaline petrochemical wastewater have been screened for the presence of protease production on Horikoshi medium supplement to skimmed milk plates. The optimum temperature for growth of isolated (code:jpc-b1) was observed 30ºC and the optimum pH:9 **(Figure 1)**.

Characteristics of the Isolated Strains

Jpc-b1 was found to be Gram-positive rod shaped bacterium, endospore-forming bacteria, aerobic, biochemical characteristics were listed in **Table 1**. Based on 16S rRNA gene analysis, the strain was phylogenetically characterized and identified the closest relative using BLAST (NCBI) search. Thus identified, the strain belonged to Bacillaceae family (**Figure 2**). Jpc-b1 displayed 99% sequence similarity with its closest relative *Bacillus sp.BAB-4161* .In this studywe present biochemical andphylogenetic data to show that isolatesjpc-b1 is new strain to name *Bacillus* sp. that 16s rRNA gene sequence was submitted to NCBI Gen Bank (ACCESSION KM214426).

Effect of pH and Time on Protease Production

Bacillus sp. Z.D.H could grow and produce protease over a range of pH (5–10). Maximum protease production was observed at pH 9 **(Figure 3)**. However majority of microorganisms producing alkaline proteases show growth and enzyme production under alkaline condition **[10]**. The effect of different temperature on protease production *Bacillus sp.Z.D.H*observed the Maximum protease production is in 48 h at 30ºC (**Figure 4**). Similar findings were also reported by some workers **[11]** in which maximum enzyme production were observed at 24 hours of growth.

Table 1: Morphological and Biochemical Characterization of *Bacillus* sp. Z.D.H Isolated from Petrochemical Wastewater

Characteristic	Z.D.H strain
Colony morphology	Large,smoth,
Gram staining	Rod, Gram posetive
Motility	Posetive
Spore staining	Negative
Catalase	Posetive
Oxidase	Negative
Starch	Posetive
Glucose	Posetive
Sucrose	Negative
Arabinose	Posetive
Mannitol	Negative
Rafinose	Posetive
Maltose	Negative
Lactose	Posetive
Growth in 6.5%	Negative
Deaminase	Negative
Indole	Negative
Citrate	Negative
Nitrate test	Posetive
H2S production	Negative

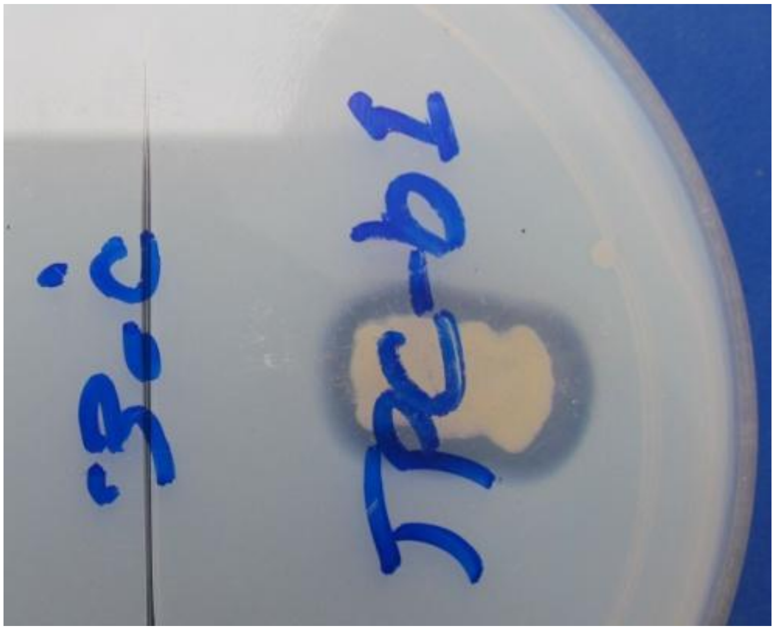

Figure 1: Proteolytic Activity of *Bacillus* sp. Z.D. Honskim Milk Agar Medium (pH 10) Incubated for 24 h at 30°C

Figure 2: Phylogenic Relationships of *Bacillus* sp. Z.D.H and Some Related Taxabased on 16S rRNA Gene Sequences. Thebranching Pattern was Generated by the Neighbour-Joining Method. The Significances for Particular Nodes were Obtained by Bootstr ap Analysis (1000 Replications). Bar, 0.1nucleotide Substitutions per site

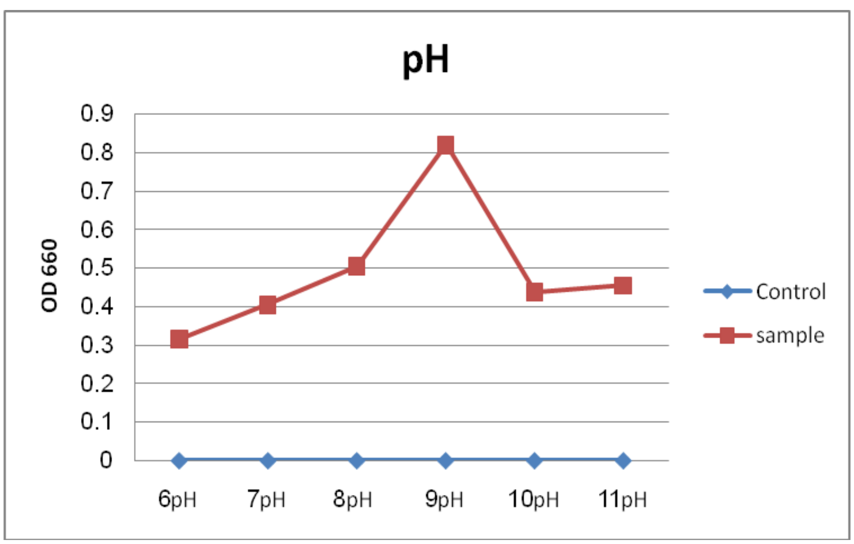

Figure 2: Effect of Various pH on Protease Production in *Bacillus sp.Z.D.H* Isolated from Alkaline Petrochemical Wastewater

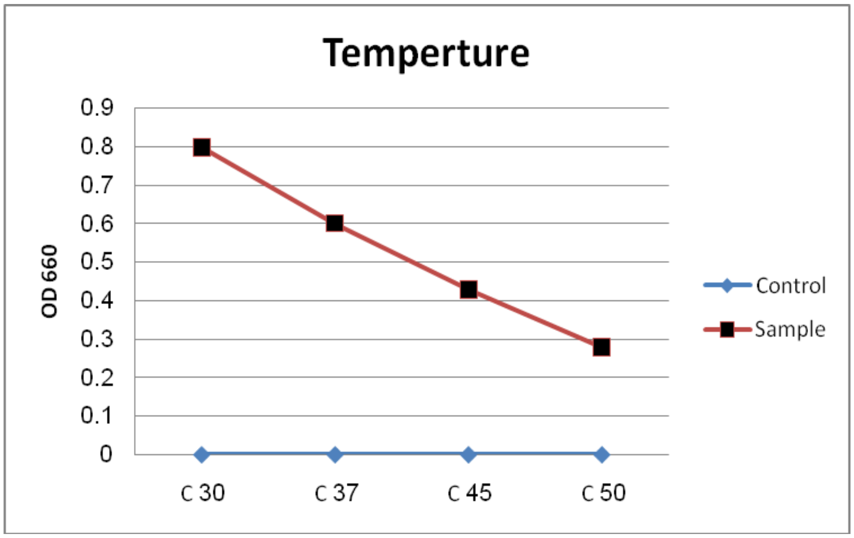

Figure 4: Effect of Various Incubation Temprature on Protease Production *Bacillus sp. Z.D.H* Isolated from Alkaline Petrochemical Wastewater

CONCLUSION

According to this study Alkaline petrochemical wastewater is a excellent source for isolated extremozymes producing bacteria. Also a good source is for the isolation of biodegradationand bioremediation bacteria and compounds dangerous pollutant and xenobiotic.

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