Isolation and Characterization of Alkaline Protease Producing Bacteria from Petrochemical Wastewater

Kavan, F., Asli Kousha H. and Heshmatipour, Z.

**Abstract**

Alkaline proteases are referring to proteolytic enzymes which work optimally in alkaline pH. Alkaline protease are produced by both neutralophilic and alkaliphilic microorganisms. This study deals with the isolation and characterization of alkaline protease from petrochemical wastewater. Isolation of alkaline protease producing bacteria was to using Horikoshi medium. After the enrichment, samples culture in different media. Maximum protease production was observed at pH 9 and 48h at 37 °C. Petrochemical wastewater due to its high content of hydrocarbons such as benzene and aromatic compounds is a promising source for the isolation of various microorganisms especially are protease producing bacteria. A gram positive bacteria was identified belong to Bacillaceae family. Then sequenced the 16s rRNA of isolates were identified by biochemical tests and the final Identification of bacteria was also sequenced 16s rRNA analysis. A gram positive bacilli is a isolate strain belong to Bacillaceae family. Then sequenced to 16 s rRNA a new strain was identified by the name Bacillus sp. Hania.ZH. Maximum protease production was observed at pH 9 and 48h at 37 °C. Petrochemical wastewater due to its chemical and alkaline compounds is a excellent source for the isolation of various microorganisms especially are protease producing bacteria.

**Introduction**

Proteases represent one of the three largest groups of industrial enzymes and account for about 60% total worldwide sale of enzymes [1]. Alkaline proteases are produced by both neutralophilic and alkaliphilic microorganisms. These two groups represent almost all sources of commercial alkaline proteases currently available in the market [2]. Alkaline proteases are referring to proteolytic enzymes which work optimally in alkaline pH. Bacterial protease has several industrial applications. It is used: as processing aid, leather tanning industries, as detergent additive, protein hydrolysis, pharmaceuticals production, and in chemical synthesis [3]. They are commercially important and isolated from various living sources such as plants, animals, bacteria and fungi [4]. Alkaliphiles are organisms isolated from extremely alkaline environments such as soda lake, having their optimum growth pH above 9 [5]. Examples of alkaliphilic microorganisms producing alkaline proteases include Bacillus firmus, Bacillus lentus, and alkaliphilic Actinomycetes [2]. Among the huge number of enzymes produced on a large scale by Bacillus species, proteases are important for diverse industrial applications [1].

The alkaline proteases from these species represent the lead molecules for the subtilisins. Members of the subtilisin superfamily of proteases have now been identified, with different functions, in practically all living organisms [6]. Although the size of subtilisins varies from 18 kDa to 90 kDa, all the subtilisins used in detergents have a size of approximately 27 kDa [7]. This study deals with the isolation and characterization of alkaline protease from petrochemical wastewater.

**Material and Methods**

Sample collection:

In this study samples were collected from petrochemical wastewater company from Tabriz, Iran. The wastewater contains multiple hydrocarbons such as benzenes compound and pH of the wastewater were 7.5. 
Isolation of alkaline protease producing bacteria:
Isolation of alkaline protease producing bacteria was to using Horikoshi medium [8] containing (g/l): Glucose 10, Polypeptone 5, Yeast extract 5, KH₂PO₄ 1, MgSO₄ 7H₂O 0.2, Na₂CO₃ 10, agar for plates 20 and supplement skimmed milk 10. Water samples inoculated in Horikoshi medium and incubated at 37 °C with aeration for 3 day to 200 rpm. After the enrichment, samples culture in Horikoshi medium supplement to skim milk. Formation of halo zone around the colonies, show is proteolyses activity. These colonies were isolated and the isolates which showed the largest halo zones were selected for further studies.

Characterization of Isolate:
The characters of the isolate were studied following the standard microbiological methods as described in Berly's Manual of Systematic Microbiology. Gram reaction, colony morphology. The physiological and biochemical characters, included: oxidase, catalase, indole, SH₂ MR, VP, tryptophane deaminase, gelatinase and ermentation oxidation of the following carbon sources.

Analyses of 16S rRNA Gene Sequences:
For the sequence analysis, bacterial genomic DNA was extracted and purified using phenol-chloroform method. Two primers annealing at the 5' and 3' end of the 16S rDNA were 27F (5'-AGAGTTTGATCMTGGCTCAG -3') and 1492R (5'-GTTACCTTGTTACGACTT - 3') [9]. PCR amplification was 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 5µl. Amplification consisted of a 1 min denaturation step at 94 °C, a minute annealing step at 59 °C and a minute extension step at 72 °C. The first cycle was preceded by incubation for 5 min at 94 °C. After 35 cycles, there was a final 10-min extension at 72 °C. 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 250 ml-glass Erlen containing 100ml of alkaline protease production medium, contained (g/l): Glucose 10, Peptone 5, Yeast extract 5, KH₂PO₄1, MgSO₄ 7H₂O 0.2, Na₂CO₃10 and incubated overnight at 180 rpm and 37 °C for 72 h. Cells and insoluble materials were removed by centrifugation at 9 000 g for 30 min at 4 °C and the cell-free supernatant was used as the source of crude alkaline protease enzyme.

Alkaline Protease Assay:
Alkaline protease activity was estimated by the method of Lowery [10]. 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 55 °C 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 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 9 at 37 °C using protease production medium to different pH.

RESULTS AND DISCUSSION
Isolation and screening of protease producing bacteria:
In the present study, a one bacteria to largest halo zones from petrochemical wastewater have been screened for the presence of protease production on Horikoshi medium supplement to skimmemilk plates. The optimum temperature for growth of isolated (code: A14) was observed 37 C and the optimum pH:9.

Characteristics of the isolated strain:
A14 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 (Fig. 1). A14displayed 98% sequence similarity with its closest relative Bacillus cereus. In this study we present biochemical and phylogenetic data to show that isolates A14 is
new strain to name *Bacillus* sp. that 16s rRNA gene sequence was submitted to NCBI GenBank (Accession No: KJ920207).

**Table 1:** Morphological and biochemical characterization of Hania. ZH strain isolated from petrochemical wastewater.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Hania. ZH strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony morphology</td>
<td>Large, irregular, rough</td>
</tr>
<tr>
<td>Gram staining</td>
<td>Short Rod, Gram positive</td>
</tr>
<tr>
<td>Motility</td>
<td>Positive</td>
</tr>
<tr>
<td>Spore staining</td>
<td>Negative</td>
</tr>
<tr>
<td>Catalase</td>
<td>Positive</td>
</tr>
<tr>
<td>Oxidase</td>
<td>Negative</td>
</tr>
<tr>
<td>Starch</td>
<td>Positive</td>
</tr>
<tr>
<td>Glucose</td>
<td>Positive</td>
</tr>
<tr>
<td>Sucrose</td>
<td>Positive</td>
</tr>
<tr>
<td>Arabinose</td>
<td>Positive</td>
</tr>
<tr>
<td>Mannitol</td>
<td>Positive</td>
</tr>
<tr>
<td>Raffinose</td>
<td>Positive</td>
</tr>
<tr>
<td>Lactose</td>
<td>Positive</td>
</tr>
<tr>
<td>Growth in 6.5%</td>
<td>Negative</td>
</tr>
<tr>
<td>Deaminase</td>
<td>Negative</td>
</tr>
<tr>
<td>Indole</td>
<td>Negative</td>
</tr>
<tr>
<td>Citrate</td>
<td>Negative</td>
</tr>
<tr>
<td>Nitrate test</td>
<td>Positive</td>
</tr>
<tr>
<td>H2S production</td>
<td>Negative</td>
</tr>
</tbody>
</table>

**Fig. 1:** Phylogenetic relationship of strain Hania. ZH (Code: A14) isolated from Petrochemical wastewater. The tree is constructed using 16S rRNA gene sequence using the Neighbour-joining method. Bar, 0.005 nucleotide substitutions per site.

**Effect of pH and Time on protease production:**

*Bacillus* sp. Hania. ZH could grow and produce protease over a range of pH (5.0-10). Maximum protease production was observed at pH 9 (Fig. 2). However majority of microorganisms producing alkaline proteases show growth and enzyme production under alkaline condition [11, 12, 13]. The effect of different time on protease production *Bacillus* sp. Hania. ZH observed the Maximum protease production is in 48 h at 37 C (Fig. 3). Under most growth conditions, *Bacillus* species produce extracellular protease during the post exponential growth phase [14]. Similar findings were also reported by some workers [15, 16] in which maximum enzyme production were observed at 48 hours of growth.
Conclusion:

In the present study, we have isolated *Bacillus* sp. Hania.ZH from petrochemical wastewater and investigated the optimal media components for highest protease production. The optimum, incubation time and pH. According to this study Petrochemical wastewater due to its chemical and alkaline compounds is a excellent source for the isolation of various microorganisms especially are protease producing bacteria.

REFERENCES


