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A Critical Analysis of Alkaline Proteases Producing Bacteria from Halophilic Soil

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Abstract

Alkaline proteases have high commercial value and find multiple applications in various industrial sectors. The present study intended to isolate a suitable bacterium for alkaline protease production. Protease producing bacteria were isolated from organic waste containing soil, screened for protease production on skim milk agar plates and confirmed the protease production through protease assay. In Study Area Rajasthan of India, almost 50% of the territory presents a predominant arid climate that includes alluvial origin principally in the arid regions, located mainly in the north of the state. The soils in Rajasthan state are typical of saline soils of the arid region of India, with the presence of alluvial and lacustrine deposits, some of them calcareous or gypsiferous. In present research study with the aim of isolating halophilic bacteria, three geographical areas of Jaipur District in Rajasthan were selected for study and were sampled (Figure 1); the areas were selected according to their saline or alkaline-sodic characteristics. In the present study, soil samples collected from Jaipur were initially analysed for Quality controls including Microbiological lab and product development. Samples were serially diluted using distilled water and spread plated on the surface of casein agar plates (nutrient agar with 1% casein) and incubated at 30^{o}C for 48hrs.

Keywords: Alkaline Protease, Fermentation, Protease assay, Bacillus Cereus, Protease Production.

Introduction

Enzymes are rightly called as the catalytic machinery of living systems, and they are playing an important role in many aspects of life since the dawn of times. In fact, they are vitally important to the existence of life itself and they help the chemical reaction take place quickly and efficiently. These nature's catalysts speed up the rates of reactions without themselves undergoing any permanent change. Except ribozyme, enzymes are protein molecules. Civilizations have used enzymes for thousands of years without understanding what they were or how they worked.

Proteases represent one of the three largest groups of industrial enzymes and have traditionally held the predominant share of the industrial enzyme market. Proteases of commercial importance are produced from microbial, animal and plant sources. They constitute a very large and complex group of enzymes with different properties of substrate specificity, active site and catalytic mechanism, pH and temperature activity and stability profiles. Industrial proteases have applications in a range of processes taking advantage of the unique physical and catalytic properties of individual proteolytic enzyme types (Ward, 1991). This vast diversity of proteases, in contrast to the specificity of their action has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications. Microbial

proteases account for approximately 40% of the total worldwide enzyme sales in various industrial market sectors, such as detergent, food, pharmaceutical, leather, diagnostics, waste management and silver recovery (Godfrey and West, 1996; Gupta et al., 2002; Sharma et al., 2001; Kumar and Takagi 1999; Rao et al., 1998; Banerjee et al., 1999; Singh et al., 2001). Proteases from microbial sources are preferred to the enzymes from plant and animal sources, since they posses almost all the characteristics desired for their biotechnological applications. Microorganisms represent an attractive source of proteases as they can be cultured in large quantities, in a relatively short time by established fermentation methods producing an abundant, regular supply of the desired product. Besides, they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications.

Review of Literature

Vadlamani and Parcha (2011) isolated 50 microbial strains from the soil samples from different regions. Among the isolates hyper producing strain namely, Bacillus clausiiwas selected for alkaline protease production. The optimum fermentation conditions of production were temperature 400C, pH 8 and time 32hrs. Fructose, peptone and Copper sulphate as good nutritional sources for producing higher yields of the enzyme.

Park and Cho (2011) found the extracellular proteolytic activity in JSP1, an Antarctic bacterial isolate. The strain was related to Bacillus sp. The JSP1 protease was partially purified by ammonium sulfate precipitation. Optimal enzyme activity occurred at 40oC and pH 7.4. Enzyme activity was significantly enhanced in the presence of Mg2+ and Ca2+ and was completely inactivated in presence of Cu2+, Zn2+, Hg2+, EDTA and SDS.

Kanmani et al., (2011) produced the protease enzyme from the bacterial strain Bacillus cereus. The strain was isolated from the estuarine sediment sample on casein agar medium. Based on the results obtained from optimization, the mass scale culture was made with pH 9.0; 40oC temperature; 35ppt of salinity; 3% of the casein as the suitable substrate. Ammonium sulphate saturation was made and the enzyme was partially purified and lyophilized. 80% saturation has given the maximum saturation of 0.9g. SDS-PAGE resulted in the separation 55 KDa and 20 KDa, both representing the enzyme protease. Application study was carried out with the enzyme recovered from Bacillus cereus. The purified enzyme was included in to the stain removal procedure. There was the better stain removing observation at the time of using the protease enzyme.

Prabhavathy et al., (2012) collected a total of seven bio-effluent samples from leather processing company in Tirupur district, TN and the bacterial strains from the effluent (B1 to B7) were screened for extracellular alkaline protease production. Among them, isolate B6 produced high activity and was identified as Bacillus subtilis by biochemical and molecular characterization. In this study, culture environment for protease production was optimized using solid state fermentation (SSF). The results revealed that wheat bran, glucose, peptone, pH 7.5, temperature of 45oC and incubation time of 96 hrs enhanced protease production.

Palsaniya et al., (2012) isolated protease producing bacteria from soil and identified them as Pseudomonas fluorescens, Bacillus subtilus, E.coli and S. marscescens. The optimum conditions for protease production were found to be 37oC at pH 10 with glucose as carbon source and peptone as nitrogen source. Soycake and calcium chloride stimulated the production of protease with 1ml of inoculum size for 48 hrs of incubation period with less concentration of EDTA. Among all studied bacterial isolates, the highest enzyme activity was observed in B. subtilis.

Narendra (2012) recovered twenty five isolates from different soil samples collected from different field nearer to Ravulapalem village, East Godavari district, Andhra Pradesh, India. On the basis of colony size, texture, and microscopic characteristics, the isolates were categorized into 10 types. Structural, staining and biochemical activity results have revealed that four of five active enzyme producing bacteria were Bacillus sp, and one was Staphylococcus sp. Protease enzyme produced was estimated for these strains.

Sinha et al., (2016) isolated a total of 23 bacterial strains from different soil samples collected from dairy farm soil and rhizospheric region of Murrayakoenigii L. sprengel (Meethi Neem). Amongst these, only five isolates showed good proteolytic activity, two isolates showed moderate activity, while other isolates showed poor activity. Maximum yield of enzyme (124.2U\ml) was obtained at 9 pH of media after 24 hrs of incubation in water bath cum shaker maintained at a temperature of 25ºC using sucrose as carbon source and yeast extract as nitrogen source. Wash performance analysis revealed that enzyme could effectively remove Indian blackberry (Syzygiumcumini L. skeels) and Banana (Musa bulbisiana) stains.

Verma and Baiswar (2018) isolated twenty three bacterial strains from treated tannery effluent of Jajmau, Kanpur, India and they exhibited variable protease activity in which one isolate TVP-9 showed a maximum clearance zone diameter of 39 mm at 37°C and 45 mm at 50°C after 24 hrs of incubation, indicated its thermostable nature and was selected as the best thermostable alkaline protease producing bacteria. The TVP-9 strain was identified as Bacillus cereus. The bacteria obtained maximum cell density at 28 hrs, whereas, it produced maximum protease of 410 Units/ ml during early stationary phase after 36 hrs of growth. The enzyme exhibited its optimal activity at pH 9.0, temperature 35-50oC and 0.0 to 2.0% salinity, whereas, significantly active and stable in broad pH (7.0-11.0) and temperature (30-60oC) range and at NaCl concentrations ranging from 0.0 to 3.0%.

Vijayaragavan et al., (2019) using agricultural residues produced protease from B. cereus strain AT. pH 8, 50oC, 72hrs incubation, 6% inoculam, 120% moisture content, maltose and yeast extract were found to be ideal at which 4813± 62U/g protease was produced. The 46 kDa protease effectively dehaired goat hides within 18 hrs of incubation at 30oC. Hasio et al., 2014 charecterized an aspartic protease from Rhizopus oryzae peptidase R, a commercial protease preparation derived from Rhizopus oryzae. It was a 39 KDa protein, which was completely inactivated by the aspartic protease inhibitor pepstatin A.

Objective of Study

- Identification of protease producing bacteria from soil Isolation and screening of protease producing bacteria from soil.
- Quantitative estimation of alkaline protease produced by bacteria.
- Optimization of the most potential strain for various physicochemical parameters

Materials and Methods

Halophilic Soil Sample Collection from Study Area

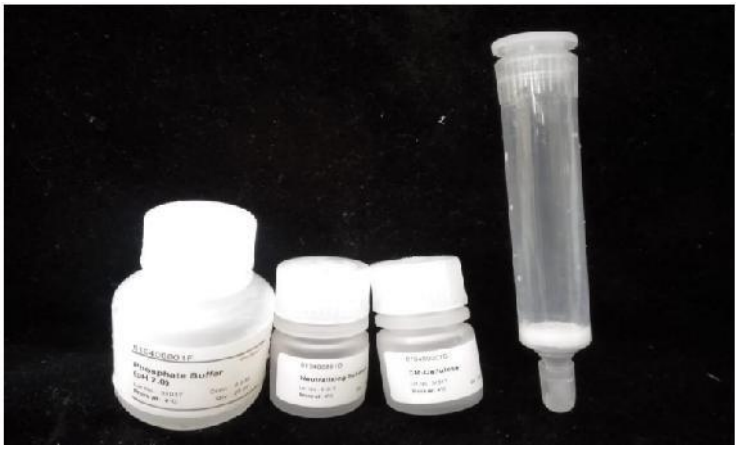
Cultivable halophilic microorganisms were isolated and identified from saline and alkaline-sodic soil from sambhar lake in jaipur. Saline and sodic soil are distributed widely around the world with a total of 932 million of hectares (ha); either natural or human-induced found three predominant environmental distributions such as arid-semiarid regions, coastal areas and humid regions (Shukla et al., 2011). Their formation is influenced by different environmental factors such as low annual precipitation, daily temperature variations, microbial activity, geological time and climate, rock weathering, inadequate

quality of irrigation, seawater intrusion onto land, mineral precipitation, dissociation of minerals, etc. All these generate a high electrical conductivity (>4 dS m−1), high exchangeable sodium percent, and sodium absorption rate (SAR) as examples of saline and sodic soil characteristics (Bui, 2013). These characteristics allow the distribution or the accumulation principally of chloride, sulphates, calcium, magnesium, carbonates, bicarbonates, and nitrates in saline soil; while in sodic soils the NaCl is the predominant salt (Singh, 2016). Particularly, in Study Area Rajasthan of India, almost 50% of the territory presents a predominant arid climate that includes alluvial origin principally in the arid regions, located mainly in the north of the state. The soils in Rajasthan state are typical of saline soils of the arid region of India, with the presence of alluvial and lacustrine deposits, some of them calcareous or gypsiferous (Krasilnikov et al., 2013).

In present research study with the aim of isolating halophilic bacteria, three geographical areas of sambhar lake in Jaipur District of Rajasthan were selected for study and were sampled (Figure 4.1); the areas were selected according to their saline or alkaline-sodic characteristics. In the present study, soil samples collected from Jaipur were initially analysed for Quality controls including Microbiological lab and product development. Samples were serially diluted using distilled water and spread plated on the surface of casein agar plates (nutrient agar with 1% casein) and incubated at 30oC for 48hrs (Naidu and Devi, 2005).

COMPOSITION OF THE NUTRIENT AGAR CASEIN MEDIUM

Ingredients	g / L
Peptic digest of animal tissue	5.00
Sodium chloride	5.00
Beef extract	1.50
Yeast extract	1.50
Casein	10.00
Agar	15.00
Distilled water	1600ml
Final pH (at 25°C)	7.4±0.2



Pictorial Representation of Collected Sample and Chemicals Isolation and Screening of Halophilic Organic Solvent Tolerant Bacteria

The halophilic organic solvent tolerant bacteria were isolated from variety of sources such as hydrocarbon contaminated soil, industrial effluents, marine resources such as marine sediments and water samples etc. Several researchers were isolated and screened different species of halophilic, organic solvent tolerant proteolytic bacterial strains from marine environment (Shivanand and Jeyaram, 2009; Annamalai et al., 2012; 2014 a, b; Sathish Kumar et al., 2015; Maruthiah et al., 2014; 2015 a, b; 2016 a, b). The

classification of Kushner and Kamekura (1998) defines different categories of halophilic microorganisms based on the optimal salt concentration; wherein, they show the optimal growth, and it includes four categories: non- halophilic organisms, defined as those require less than 1% NaCl; whereas, if they can tolerate high salt concentrations, then they are considered as halotolerant microorganisms.

Considering the significance of Halophilic proteolytic microorganisms, it has been explored from various resources such as Veraval costal region (Gujarat), Kandla salt pans (Gujarat), Kumta coast (Karnataka), Alang coast (Gujarat), Sambhar Salt Lake (Rajasthan), Somnath coast (Gujarat), Alibagh coast (Maharashtra), Lonar Lake (Maharashtra), Parangipettai coast (Tamil Nadu), and Rajakkamangalam coast (Tamil Nadu). The most common bacterial genera recorded in these coastal environments include Haloarcula, Halobacteria, Halomonas, Geomicrobium, Flavobacterium and Bacillus (Annamalai et al., 2012; 2014 a, b; Maruthiah et al., 2014; 2015 a, b; 2016 a, b)

Among the above mentioned bacterial isolates, most of them were reported to have both halophilic and organic solvent tolerant capacity. In this perspect, Inoue and Horikoshi (1989) reported the first organic solvent proteolytic bacterium (Pseudomonas putida IH200) with maximum growth and protease activity in toluene enrichment medium. Followed by this, various researchers isolated different organic solvent tolerant bacterium in the presence of solvent enrichment medium.

Screening for Proteolytic Activity

The isolated bacterial strains were purified on casein agar plates and they were inoculated into casein broth (i.e.) nutrient agar with 1% casein. After the incubation period culture filtrates were separated by centrifuging at 8000 rpm for 15min. The culture filtrate thus prepared was used for the qualitative protein production assay. Water agar medium of Carrim et al., 2006 (1.8g agar in 100ml of dist. water) supplemented with 1% casein was poured in to petri plates and after solidification 8 mm diameter wells were made using a cork borer. 100µl of culture filtrates were inoculated each one in a separate well and a same volume of uninoculated broth was poured in a separate well as control. After 24 hrs of incubation, enzyme activity was visualized as a clear zone on addition of 1% mercuric chloride solution in 1N HCl. The diameter of zone formed was determined for all the positive strains.

Optimization

The cultural factors such as inoculm concentration, agitation, pH, temperature, salinity, carbon and nitrogen sources which are expected to affect the production of protease by the selected strain were optimized by selecting one parameter at a time. Unless otherwise mentioned, nutrient broth medium was used throughout the optimization study.

- **Inoculum Concentration**

Based on screening the most potent protease production strain was identified as Bacillus cereus and it was designated as B. cereus. It was inoculated on nutrient agar slant and incubated at 30ºC. From the slant, a loopful of culture was inoculated into nutrient broth supplemented with 1% casein. This was treated as the pre-inoculum or mother culture. The inoculated, pre- inoculum culture flask was incubated in a rotary shaker at 150 rpm at 30ºC for 18 - 24 hrs. 1-3% of inoculum was tested to determine the optimum concentration.

- **Static and Shaken Condition**

Effect of agitation on growth and protease production by un-agitated (Static) culture and the culture agitated at different levels viz., 50, 75, 100, 125, 150, 175 and 200 rpm was determined. Relative activity was estimated as mentioned previously.

- **pH**

Different pH ranging from 6-11 (at an interval of pH 1) were maintained in the medium and incubated. Growth and enzyme activity were assessed for every 6hrs until 48 hrs.

- **Temperature**

Different temperatures such as 25oC, 30oC, 35oC, 40oC and 45oC at an interval of 5oC were tested for growth and enzyme activity and assessed for every 6hrs up to 48 hrs.

- **NaCl Concentration**

Different salinity ranging from 0 – 3.5% (at the interval of 0.5%) were maintained in the medium and incubated. Growth and enzyme activity were assessed for every 6 hrs up to 48 hrs.

- **Carbon Sources**

Different carbon sources such as glucose, maltose, Fructose, sucrose and starch were added in the medium in separate tubes at the concentration of 1% and incubated. Growth and enzyme activity were assessed for every 6hrs up to 48hrs.

- **Concentration of Ideal Carbon Source**

Different concentration of glucose as carbon source (0.5 – 2.5%) was maintained in the medium and incubated. Growth and enzyme activity were assessed for every 6 hrs up to 48hrs.

- **Nitrogen Sources**

Nitrogen sources such as beef extract, yeast extract, peptone, ammonium nitrate, Ammonium sulphate and potassium nitrate were added in the medium separately at the concentration of 0.5% in the medium and incubated. Growth and enzyme activity were assessed for every 6 hrs up to 48hrs.

- **Concentration of Ideal Nitrogen Source**

Different concentration of yeast extract as nitrogen source (0.1-1.0%) was maintained in the medium and incubated at 35oC. Growth and enzyme activity were assessed for every 6 hrs up to 48hrs.

Statistical Analysis

Statistical analysis was run using Stat graphics Centurion XVI version 16.1.18 software (Stat graphics Centurion for Windows, Stat point Technology, Inc., USA). Analysis of variance (ANOVA) of each variable of soil characterization was performed by a completely randomized block design, where each block was the soil sample. Block means were compared using the least significance difference (LSD) multiple range test, calculated at 0.05 probability level ($P < 0.05$). Principal component analysis (PCA), a conventional multivariable technique, was performed for soil physicochemical variables to identify groups of variables contributing most to microbial diversity between study areas. PCA is based on the correlation (covariance) matrix, which measures the interrelationships among multiple variables.

Results

Screening for Proteolytic Activity

From casein agar plates 151 strains of varying morphology are selected and screened for proteolytic activity adopting well assay method. The zone of clearance activities are measured and found to be in the range of 4mm-15mm. As most of the strains showed activity with 4mm range, 5 potential strains are

selected for the further study. Among the five, the one with 15mm of zone was selected for protease production.

Optimization

In the present study five proteolytic bacterial strains identified, and they B.cereus, E. coli, B.subtilis, B. aeruginosa and B. Pumilis among the identified proteolytic strains, B.cereus shed maximum protease production and hence it is stain was also selected for optimization, characterization and application studies.

- **Inoculum Concentration**

When the log phase culture of B. cereus was tested for the suitable inoculum concentration in the range of 0.5 – 4%, 1% inoculum resulted in the maximum OD value of 1.202. On further increase in concentration of inoculum decrease in growth of the culture in shake flask are noted. Likewise the protease production also found to be the maximum at this inoculum concentration (1284U/ml/min.). Surprisingly at 4% inoculum concentration growth is reduced to 0.88 OD at which enzyme production was found to be only 4U/ml/min.

- **Static and Shaking Conditions**

The effect of agitation test at the range of 50 – 200rpm. At 50rpm the OD value is found to be 0.6 which was in increasing trend on further increase up to 150rpm, where OD value of 1.202 is observed. Further increase in agitation is reduced and the growth and value of enzyme activities are found at 0.89 and 800 U/ml/min. observed at 200 rpm. However least growth and enzyme activity found when incubation done in static condition. When cultures are kept static growth attained the level of 0.442 OD, which resulted in only 250 U/ml/min. of enzyme activity.

- **pH**

When a pH range of pH 6 to pH 11 is tested, pH 10 resulted in higher OD as well as higher enzyme activity. A maximum of 1249 U/ml/min. Activity is observed at pH 10 at 46 hrs where it is minimum of 802 U/ml/min. at pH 8, 1041 U/ml/min. at pH 9 and 981 U/ml/min. at pH11. However it is only 561 U/ml/min. at pH 7 at 46 hrs of incubation.

- **Temperature**

At 45oC maximum OD value of 1.05 is obtained in which the protease activity is observed at 1057/ml/min. At the end of 42 hrs protease production reduced and the activities are found to be 997U/ml min. with a growth of 0.95 OD. Lower growth and enzyme activity is observed at both extremes (i.e.) 25oC and 45oC .

- **NaCl Concentration**

When NaCl concentration of 0 to 2% is tested at an interval of 0.5%, the maximum growth and enzyme activity is observed at 0.5% NaCl. Maximum OD value of 1.14 with an enzyme activity of 1092 is obtained at 46 hrs at that concentration. The minimum is observed at 2% NaCl with an OD value of 0.78 at 46 hrs, at which only 524 U/ml/min. of enzyme activity is observed. Irrespective of concentration after 46 hrs both OD value and enzyme production were decreased.

- **Carbon Sources**

In the present study, to select a potential carbon source, glucose, maltose, fructose, sucrose and starch is incorporated in separate flasks at 1% concentration. Among them glucose favoured the maximum growth and protease production respectively with 1.4 OD and 1012U/ml/min. The minimum growth (0.8 OD) and enzyme production (587U/ml/min.) is observed when starch is used as the sole carbon source.

Scope for Future Research Work

The novel soil bacterial isolates are isolated and studied as lipase producing bacterial isolates. So there lies a great amount of work to be explored and performed for their characterization and unknown properties in the following terms.

- The lipase enzyme protein profiling may be carried out to characterize the lipase enzyme in terms of its structure, molecular weight and peptide sequence.
- These novel bacterial strains are also noted to possess the bio-surfactant activities on various vegetable oils used under the study and hence they can further be studied for these biosurfactant properties that may lead to the possible derived products and for the applications in industrial processes and academic research.
- These novel bacterial isolates can also be studied for expression and extraction of other industrially important enzymes like proteases, amylases, cellulases etc.
- Other industrial applications like, in degradation of xenobiotic materials; treatment of effluents containing various organic matter; production of antibiotics etc. can also be explored for the efficient use of these novel bacterial isolates.

Conclusion

Thus in the present study a soil originated B. Cereus strain is characterized and it is found to be an ideal producer of alkaline protease. This study also revealed the potential for the industrial scale protease production using this strain. The abundance of protease producers in halophilic soil sample also indicated it as a new source for the search of industrially important alkaline proteases. Enzymes are the best catalysts known for their high efficiency in biosystems and in many of the emerging industrial processes. Lipase is one such enzyme having the great potential for the conversion of lipid molecules in and out of the biological systems and thus help the environment to sustain the life from the time immemorial. This vital property of alkaline protease has been recognized and been explored for many of the industrial processes of dairy products, detergents, textiles, leather and so on to serve the mankind. In this endeavour these enzymes extracted and produced from various biological sources and one of such sources being the bacteria has made the research to be focused in search of novel bacterial strains. In addition, the growing patented bacterial strains and the degenerative bacterial strains gave the impetus for the search of novel bacterial sources for the better and improved enzyme production to meet the growing demand for bacterial species.

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