Isolation and Identification of Extracellular Alkaline Protease Producing Bacteria from Soil Sample and Enzyme Immobilization

N.S. GUPTA

Department of Biotechnology, Amity Institute of Biotechnology, Amity University, Lucknow, Uttar Pradesh, India, Corresponding Author: publication.cytogene@gmail.com

Abstract

In this study two extracellular alkaline protease enzyme producing bacteria Neisseria veillonella and Azotobacter were isolated from two different soil sample, collected from Lucknow, India and Pokhara, Nepal region. On the basis of phenotype, biochemical test, temperature, pH, carbon, nitrogen source optimization both strain were identified. Both strains were most stable at high alkaline medium but Azotobacter was comparatively less stable at room temperature. Urea was most stable nitrogen sources for both bacterial strains.

Keywords: Alkaline protease, Azotobacter, Neisseria, extracellular enzyme, immobilization.

Introduction

Alkaline protease is widely used as a major part of the whole world enzyme production which is about 40%, with constantly increasing demand because of its applications in bakery, brewing, detergent, diagnostic reagents, feeds modification, leather finishing, laundry additives, pharmaceuticals, peptide synthesis, silk, silver recovery from X-ray or photographic film, soy processing, and waste treatment [1]. Protease hydrolyses the proteins by cleavage of peptide bond [10, 11]. Alkaline protease is most stable and active even at high alkaline range therefore, they have so many biotechnological applications [12]. Microorganisms are important biological resources for enzyme production because of their characteristics like extensive biochemical diversity, possibility of easy mass culture and ease of genetic operations, storage, transfer, etc. Due to these specific characteristics present in microbes these are now known to play a major role in the production of both extracellular and intracellular enzymes on large scale, and there are more than 3000 different types of microbial extracellular enzymes that have been isolated [6]. Bacteria were preferred as comparison to other microbes because of their specific characteristics like rapid growth, easy sub culturing and revival, long time storage and readily accessible for genetic operations [8, 9]. Enzyme immobilization is an effective approach for the stabilization of enzyme as well as a useful approach for the reusability of enzyme, bioprocess control, and simplifying product separation [2, 7]. Modern and developing biotechnology using immobilized biocatalysts has recently gained the attention of many biotechnologists. Immobilized enzymes or whole cells are known for large applications due to better stability [3, 4] high efficiency of catalysis [5] and reusability.

Experimental Work

Two soil samples were collected; one from Indira Nagar, Lucknow, India which was labeled as Sample 1 and the other sample was from Pokhra, Nepal which was referred as Sample 2.

Isolation of Bacteria

Nutrient agar media was prepared for the growth of soil samples bacteria. Samples were serially diluted to minimize the concentration of bacteria. Finally, 10^{-7} diluted sample was used for the spreading on plates and was incubated for 24 h at 37°C. After 24 hours a fresh plate was prepared for the growth of specific bacteria using streak method and was incubated for overnight at 37°C. Bacterial strains were identified by gram staining.

Screening of Bacteria

Biochemical Tests

For Indole test, tryptone broth was prepared and both soil bacterial cultures were inoculated. After incubation at 37°C for 24 hours 5 drops of Kovác's reagent was directly added to the tube. A positive test was indicated by the formation of a pink to cherry red ring. In MR-VP test both bacterial strains were inoculated in glucose phosphate medium and incubated at 37°C. Then few drops of 0.04% of methyl red, VP-1 and VP-2 reagent in MR-VP respectively were added, red coloured ring indicated positive result. To find out the sugar fermenting properties, test was done in three different tubes containing different types of sugar broth viz., glucose, sucrose and lactose respectively, inoculated with cultured bacteria in aseptic condition at 37°C for 24 hours. In case of sugar fermenting positive bacteria the pink colour of the sugar broth tube changed into yellow colour. Both bacterial strains were streaked on casein agar media, bacteria that produced a caseinase enzyme degraded the casein in media, zone formation was observed. Nutrient agar media was used to find out the catalase activity of bacteria. Catalase positive bacteria converted the H_2O_2 to water and oxygen. In urease test bacteria were inoculated in urea broth medium, containing a pH indicator phenol red. In case of positive bacteria urease production in medium changes the colour from red to deep pink. Amylase activity of the bacteria was identified by streaking on starch medium. Bacteria that produced alpha amylase degrading the starch were identified as positive bacteria by adding iodine solution; clear zone was observed [15].

Isolation of protease producing bacillus species

Samples were inoculated on skim milk agar plates containing peptone (0.1%), NaCl (0.5%) and skim milk (10%) prepared using sea water and then incubated at 28±2°C for three days [14].

Purification of protease

For the purification of extracellular protease, both strains were inoculated in 50 ml of protease specific medium broth containing glucose 5.0 g/L, peptone 7.5 g/L, $MgSO_4.7H_2O$ 5.0 g/L, KH_2PO_4 5.0 g/L, and $FeSO_4.7H_2O$ 0. 1 g/L, pH was adjusted to 7.0 and the culture was incubated in a rotary shaker at 180 rpm at 28°C for 3 days. The whole fermentation broth was centrifuged at 10,000 rpm for 5 minute at 4°C and the clear supernatant was recovered and stored as crude enzyme [15].

Enzyme immobilization

Calcium chloride solution (1.4%) was prepared along with a solution of Sodium alginate (4%). 100µl of crude enzyme extract was added in sodium alginate solution and with the help of micropipette, beads formation was occurred.

Results and Discussions

Sample 1 was identified as a gram negative bacterium, which hydrolysed casein but did not degraded tryptophan. The bacterium showed positive results of catalase test, urea test, starch hydrolysis sugar fermentation and MR-VP test. Sample 2 bacteria was identified as gram negative bacteria, positive result were observed for casein test, catalase test, urease test, starch hydrolysis test, MR-VP and sugar fermentation test, however, for indole test negative result were observed. According to the results of Lowry's test, the concentration of the sample 1 was obtained as 0.9341µg/ml at an absorbance of 0.40 and the concentration of the sample 2 was obtained as 1.6743µg/ml at an absorbance of 0.79.

Bacterial strain optimization was done based on pH, temperature, carbon and nitrogen. According to the results the highest stability and the maximum bacterial growth was observed in alkaline medium at pH 10, 35°C temperature, lactose as carbon source and urea as nitrogen source in comparison to other sources. According to the study conducted on *Bacillus subtilis* the optimum condition was shown at pH 9.0, 40°C temperature, glucose as carbon source and beef extract as nitrogen source [16]. According to another study conducted on *Streptomyces ambofaciens* the optimum condition was shown at pH 8.5, 30°C temperature, starch as carbon source and yeast extract as nitrogen source [17]. A detailed study conducted on several bacterial species such as *Pseudomonas* sp, *Bacillus subtilis*, *E.coli*, *Serratia* sp and *Bacillus cereus* showed that the optimum conditions were observed at pH 9.0, 37°C temperature, glucose as carbon source and yeast extract and beef extract as nitrogen source [18]. Many more studies also revealed similar results such as marine Bacillus sp. at pH 7.0, 30°C temperature, starch or maltose along with wheat bran as carbon source and yeast extract as nitrogen source [19] and a different study on Bacillus sp. showed optimum conditions at at pH 9.0, 25°C temperature, sucrose as carbon source and yeast extract as nitrogen source [20]

Table1: Staining and biochemical testing

S.No.	Biochemical Test	Observation	Sample 1	Sample 2
1	Gram Staining	Pink colour	-	-
2	Casein Hydrolysis Test	Clear zone appeared	+	+
3	Indole Test	No Ring	-	-
4	Catalase Test	Bubbles evolved	+	+
5	Urease Test	No deep pink colour	-	+
6	Starch Hydrolysis	Dark bands appeared	+	+
7	MR-VP	Ring appeared	+	+
8	Sugar Fermentation	Pink to yellow colour change	+	+

Table 2: Lowry's Assay

Test tubes	Vol. of std. BSA (µl)	Vol. of distilled water (µl)	Conc. of BSA (mg/ml)	Vol. of reagents D	Incubation temperature	Vol. of FC reagent	OD 600nm	
							Sample 1	Sample 2
1	500	500	100	5 ml	Room temperature	0.5 ml	1.09	0.79
2	600	400	120	5 ml		0.5 ml	1.39	1.09
3	700	300	140	5 ml		0.5 ml	1.70	1.40

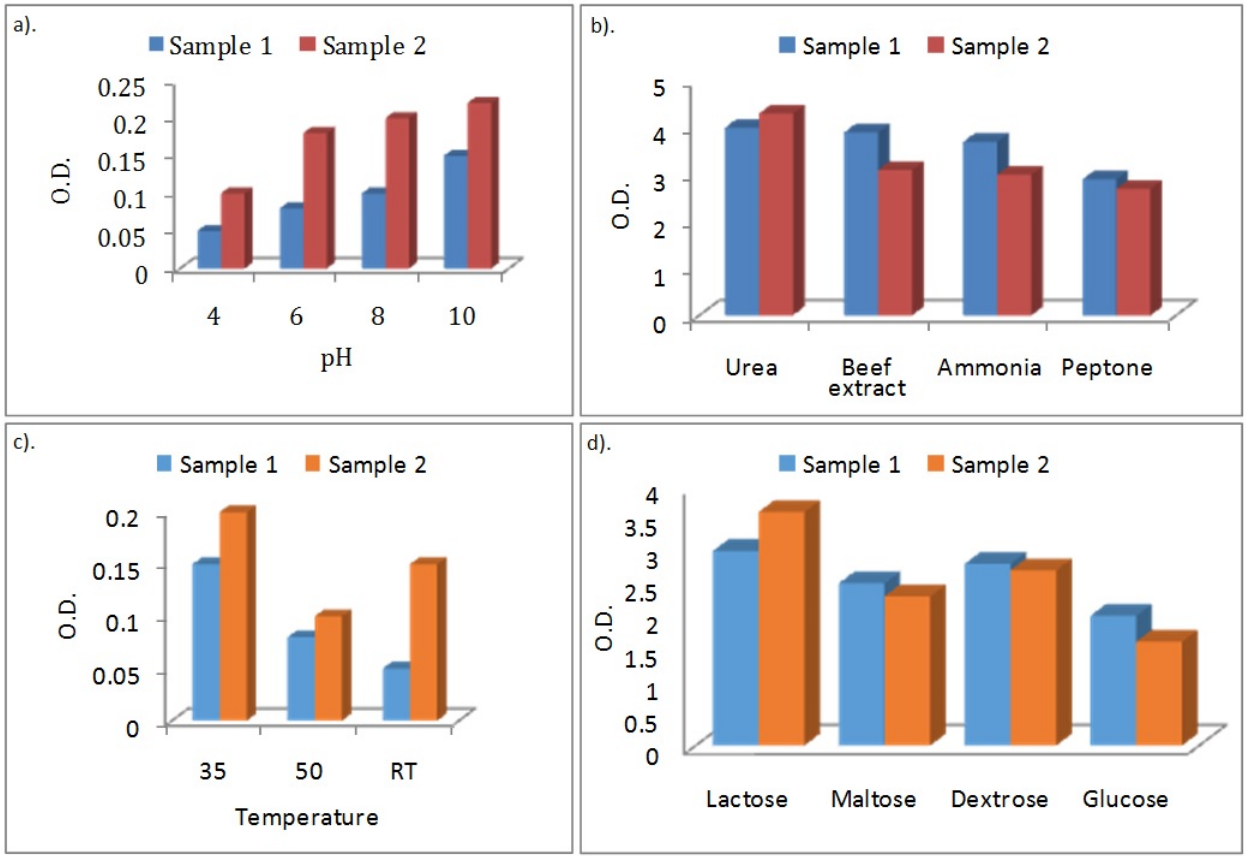

Fig.1: Bacterial strains optimization tests; a). pH, b). Nitrogen source, c). Temperature, d). Sugar source

Conclusions

The present study concludes that after gram staining and biochemical tests two bacteria *Neisseria veillonella* and *Azotobacter* were isolated from soil sample 1 and 2, respectively. Extracellular alkaline protease producing properties was identified by skim milk agar plate, due to optimization on different temperature and pH. Alkaline protease has an optimum pH greater or equal to 10 and they are most stable at high temperature.

References

[1] R. Gupta, Q. Beg, and P. Lorenz, "Bacterial alkaline proteases: molecular approaches and industrial applications," Appl. Microbiol. Biotechnol., vol. 59, pp. 15–32, 2002.

[2] C. Mateo, J. M. Palomo, G. Fernandez-Lorente., J. M. Guisan., R. Fernandez-Lafuente, "Improvement of enzyme activity, stability and selectivity via immobilization techniques," Enzym. Microb. Technol., vol. 40, pp. 1451–1463, 2007.

[3] C. Fortin., J.C. Vuillemard, "Culture flourescence monitoring of immobilized cells. In: Bont JAM, J Visser, B Mattiasson, J Tramper, eds. Physiology of immobilized cells, Elsevier, pp. 45-55, 1990.

[4] T. Kukubu., I. Karube., S. Suzuki, "Protease production by immobilized mycelia of Streptomyces fradiae," Biotechnol. Bioeng., vol. 23, pp. 29-37, 1981.

[5] S.V. Ramakrishna., R. Jamuna., A.N. Emery, "Production of ethanol by immobilized yeast cells," Appl. Biochem. Biotechnol., vol. 37, pp. 275-282, 1992.

[6] R. Patel., M. Dodia., S. Singh, "Extracellular alkaline proteases from a newly isolated haloalkaliphilic Bacillus sp.: production and optimization," Process. Biochem., vol. 40, pp. 3569–3575, 2005.

[7] R. S. Kumar, G. Ananthan, A. S. Prabhu, "Optimization of medium composition for alkaline protease production by Marinobacter sp. GA CAS9 using response surface methodology - A statistical approach," Biocataly. Agric. Biotechnol., vol. 3, pp. 191–197, 2014.

[8] B. W. Lee, B. K. Kim, Y. J. Lee, C. H. Chung, J. W. Lee, "Industrial scale of optimization for the production of carboxymethylcellulase from rice bran by a marine bacterium, Bacillus subtilis subsp. Subtili A-53," Enzym. Microb. Technol., vol. 46, pp. 38–42, 2010.

[9] N. Annamalai, M. V. Rajeswari, R. Thavasi, S. Vijayalakshmi, T. Balasubramanian, Solvent stable protease from Bacillus halodurans CAS6 using marine shellfish wastes: a potential additive for detergent and antioxidant synthesis," Bioproc. Biosyst. Engineer., vol. 7, pp. 873–883, 2013.

[10] R. Abdullah, H. Ashraf, I. Haq, "Optimization and kinetic analysis of carbon sources on the production of alpha amylase by *Saccharomyces cerevisiae*," J. Food. Technol., vol. 1, pp. 187-190, 2003.

[11] K. Ohdan, T. Kuriki, H. Takata, S. Okada, "Introduction of raw starch-binding domains in to Bacillus subtilis alpha amylase by fusion with the starch-binding domain of Bacillus cyclomaltodextrin glucanotransferase," Appl. Environ. Microbiol, vol. 66, pp. 3058-3064, 2000.

[12] A. Haddar, R. Agrebi, A. Bougatef, N. Hmidet, A. Sellami-Kamoun, M. Nasri, "Two detergent stable alkaline serine-proteases from Bacillus mojavensis A21: Purification, characterization and potential application as a laundry detergent additive," Bioresour. Technol., vol. 100, pp. 3366-3373, 2009.

[13] H. Vashist, D. Sharma, A. Gupta, "A review on commonly used biochemical test for bacteria," vol. 1, pp. 1-7, 2013.

[14] F.Uyar, I. Porsuk, G. Kizil, E. Yilma, "Optimal conditions for production of extracellular protease from newly isolated Bacillus cereus strain CA15," EurAsia. J. BioSci., vol. 5, pp. 1-9, 2011.

[15] S.F. Josephine, V.S. Ramya, D. Neelam, B.G Suresh, K. G. Siddalingeshwara, N. Venugopal, T. J. Vishwanatha, "Isolation, production and characterization of protease from Bacillus Sp isolated from soil sample," Microbiol. Biotech. Res., vol. 2, pp. 163-168, 2012.

[16] N.S. Nisha, J. Divakaran, "Optimization of alkaline protease production from *Bacillus subtilis* NS isolated from sea water," Afr. J. Biotechnol., vol. 13, pp. 1707-1713, 2014.

[17] N. A. M. Abdelwahed, Enas N. Danial, Noura El-Ahmady El-Naggar, Asem A. Mohamed, "Optimization of alkaline Protease production by *Streptomyces ambofaciens* in free and immobilized form," Am. J. Biochem. Biotechnol., vol. 10, pp. 1-13, 2013.

[18] N. S. Nisha, J. Divakaran, "Optimization Parameters for Alkaline protease Production using Bacterial isolates from different coastal regions of Tamil Nadu, India," Int. J. Curr. Microbiol. App. Sci., vol. 3, pp. 500-505, 2014.

[19] M. K. Gouda, "Optimization and purification of Alkaline Proteases produced by Marine Bacillus sp. MIG isolated from Eastern Harbour of Alexandria," Polish Journal of Microbiology, vol. 55, pp. 119-126, 2006.

[20] P. Sinha, R. K. Singh, R. Srivastva, R. Sharma, S. P. Tiwari, "Characterization and optimization of alkaline protease enzyme produced by soil borne bacteria," Trends in Life Sciences, vol. 2, pp. 38-46, 2013.