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ISOLATION AND OPTIMIZATION OF PROCESS PARAMETERS FOR ALKALINE PROTEASE PRODUCTION USING *BACILLUS SPP.*

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ABSTRACT

Soil samples were used to isolate different protease producing bacteria. Casein hydrolysis media was used to screen proteolytic bacteria which produced clear hydrolyzed zone. Isolates showing positives result for all isolates. The media (MD8) indicated maximum protease production for *Bacillus cohinii.* Glucose showed maximum enzyme activity of 14.49 U/ml for BS1. In nitrogen source , ammonium chloride showed to be best with enzyme activity as 7.85 U/ml for BS1 in MD5 media. MD5 media supplemented with NaCl works best for alkaline protease production. But a concentration of 11% NaCl showed maximum activity with 10.87 U/ml for BS1. It was found that all the isolates showed maximum protease production at 45°C in MD5 media.

Keywords : *B. cohinii, proteolytic bacteria, optimization, alkaline protease.*

Proteases are proteolytic enzymes that catalyze the breakdown of proteins by hydrolysis of peptide bonds. Proteolytic enzymes are ubiquitous in occurrence, being present in all living organisms and are essential for cell growth and differentiation. Proteases can be produced from wide diverse sources such as plants, animals and micro-organisms. The majority of commercial alkaline proteases are produced by bacteria, especially Bacillus sp. [4]. Several *Bacillus* species involved in protease production are e.g., *B. cereus, B. sterothermophilus, B. mojavensis, B. megaterium and B. subtilis* [4, 8]. Proteases are also useful and important components in biopharmaceutical products such as contact-lens enzyme cleaners and enzymatic deriders [6]. The proteolytic enzymes also offer a gentle and selective debridement, supporting the natural healing process in the successful local management of skin ulcerations efficiently.

About 35% of total microbial enzymes used in detergent industry are derived from bacterial sources and most of them produced by Bacillus spp. [2]. Many species of Bacillus have been reported to produce extracellular proteases which are used in food, brewing and laundry industry. These include *Bacillus amyloliquefaciens* [5], *B. megaterium* [7], *B. subtilitis* [9], *B. stearothermophilus* and *B. themoproteolyticus* [1]. The present study was aimed to isolate alkaline protease producing bacteria from soil and to optimize process parameters for alkaline protease production.

MATERIALS AND METHODS

Isolation and Screening of Protease producing bacteria Soil samples were used to isolate bacteria by serial dilution method [10].

Identification of the microorganism using different tests : The isolates were identified by staining Gram staining, and biochemical tests like Sugar Fermentation, citrate utilization test, catalase test, MR/VP test, urease test, starch hydrolysis test, and indole test [10].

Fermentative production alkaline protease : A loopful from bacterial culture was transferred into 2 ml NAM medium and incubated for 48 hours. These inoculums was then transferred to 10 ml of fermentation medium, incubated for 48 hours and finally transferred to 100 ml medium. These inoculums were further used for the entire study at a rate of 5% for enzyme production. The enzyme production media was prepared in 250 ml Erlenmeyer flask, sterilized and incubated for 72 hours on shaking incubator at 25 to 30 rpm.

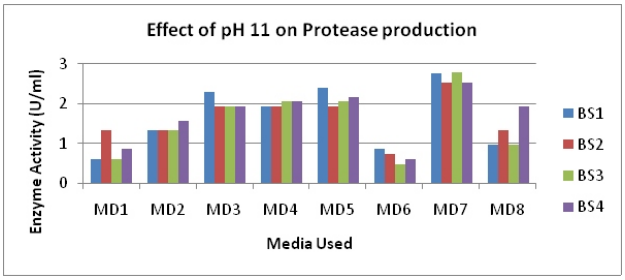
Optimization of process parameters for alkaline protease production : Optimizations of various parameters were performed for pH, carbon source, nitrogen source, salt concentration, metal ions, and temperature for five isolated species of *Bacillus*. All the parameters were optimized for eight enzyme production media (MD1-MD8).

RESULTS AND DISCUSSION

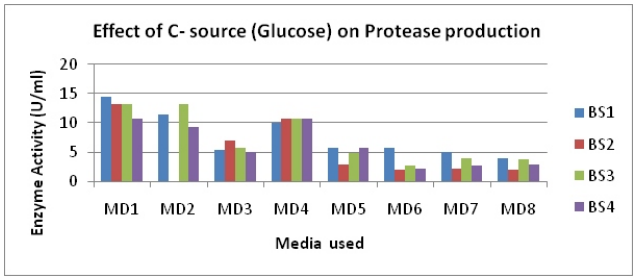
Isolation of Bacteria from soil : Isolation of bacteria was done from soil serially diluted upto 10^{-7} fold and spread onto nutrient agar media. Five different bacterial species were isolated, marked and indicated as BS1, BS2, BS3, BS4 and BS5 for further study.

Identification of Isolated Bacterial Species : Identification of the isolated bacterial species were performed by biochemical tests and comparing the results with standard description given in Bergey's Manual of Systematic Bacteriology.

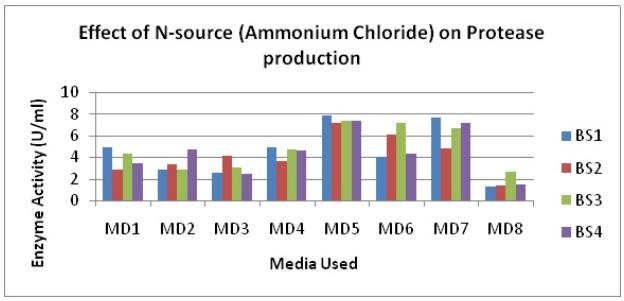
Gram Staining : The bacterial species were stained with Gram's stain in order to study its morphology and cell wall



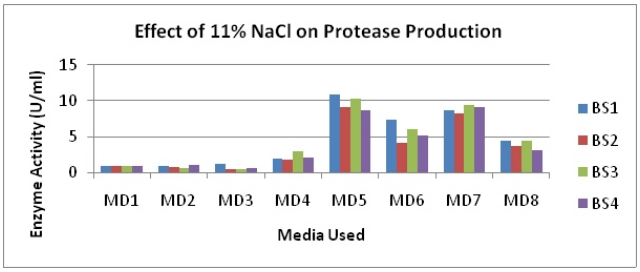
*BS1-Bacterial species 1; BS2- Bacterial species 2; BS3-Bacterial species 3; BS4- Bacterial species 4. Figure 2: Effect of pH on Alkaline Protease production by four different *Bacillus Spp.*



*BS1-Bacterial species 1; BS2- Bacterial species 2;BS3-Bacterial species 3; BS4- Bacterial species 4. Figure 3: Effect of C-source on Alkaline Protease production by four different *Bacillus Spp.*



*BS1-Bacterial species 1; BS2- Bacterial species 2;BS3-Bacterial species 3; BS4- Bacterial species 4. Figure 4: Effect of N-source on Alkaline Protease production by four different *Bacillus Spp.*

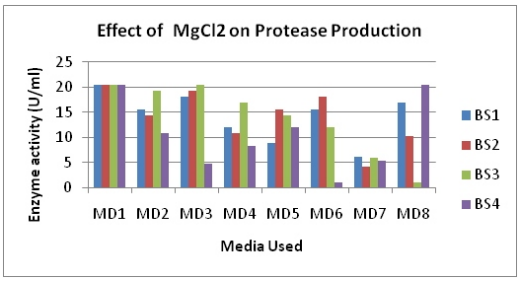


*BS1-Bacterial species 1; BS2- Bacterial species 2;BS3-Bacterial species 3; BS4- Bacterial species 4. Figure 5: Effect of N-source on Alkaline Protease production by four different *Bacillus Spp.*

type. All the bacteria were found to be Gram's positive and rod shaped.

BIOCHEMICAL TESTS

Optimization of process parameters for alkaline protease production : The media (MD8) with basal components sucrose, casein, yeast extract, K_2HPO_4, $MgSO_4$ and $NaSO_4$ indicated maximum protease production for *Bacillus cohinii*. Here at pH-11, BS1, BS2, BS3,BS4 all showed maximum protease production in MD7 -2.77 U/ml, 2.53 U/ml, 2.78 U/ml and 2.54 U/ml respectively and minimum enzyme activity was seen at pH 7 (neutral pH) as shown in Figure-2.



*BS1-Bacterial species 1; BS2- Bacterial species 2; BS3-Bacterial species 3; BS4- Bacterial species 4. Figure 6: Effect of metal ion on Alkaline Protease production by four different *Bacillus Spp.*

In case of glucose, source was optimized to be best suited carbon source in alkaline protease production. It showed maximum enzyme activity of 14.49 U/ml for BS1in MD1, 13.28 U/ml- BS2 and BS3 and 10.87 U/ml for BS4. Sucrose showed a poor turnover of alkaline protease production. Lactose was the second best carbon source which showed best enzyme activity up to 10.87 U/ml as shown in Figure-3.

Among the four nitrogen sources- Gelatin, urea, ammonium chloride and ammonium nitrate-ammonium chloride showed to be best nitrogen source with a enzyme activity as high as 7.85 U/ml for BS1 in MD5 media, MD7 also showed a significant amount of protease production by BS1 (7.61 U/ml), BS3 (6.64 U/ml) and BS4 (7.12 U/ml) as shown in Figure-4.

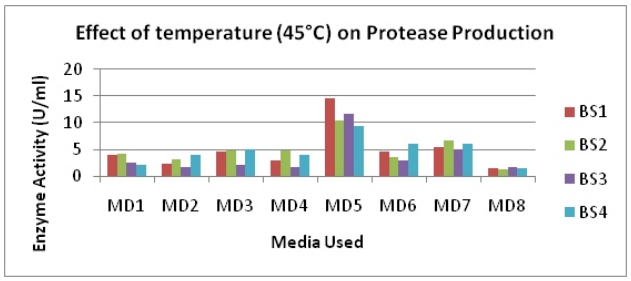
This study also shows that NaCl can add to enhanced enzyme activity for alkaline protease production. MD5 media supplemented with NaCl works best for alkaline protease production. But a concentration of 11% NaCl showed maximum activity with 10.87 U/ml for BS1. BS2 (9.18 U/ml), BS3 (10.38 U/ml) and BS4 (8.81 U/ml) also shows enhancing effect of NaCl on protease production. This study also shows that media supplemented with Mg^+ ions will elevate the protease production (20.53 U/ml) as compared to other metal ions like potassium, mercuric and manganese as shown in Figure-5.

Optimization at different temperature concluded that all the isolates showed maximum protease production at 45°C IN MD5 media. The highest production was observed for BS1(14.49 U/ml), followed by BS3 (11.59

Table-1 : Biochemical test results of BS1, BS2, BS3, BS4 and BS5

S. No.	Biochemical tets		Results				
			BS1	BS2	BS3	BS4	BS5
1.	Gram staining		Gram +ve	Gram +ve	Gram +ve	Gram +ve	Gram +ve
2.	Morphology		R	R	R		R
3.	Sugar Fermentation	Glucose	+ve	+ve	-ve	+ve	-ve
		Sucrose	+ve	-ve	-ve	+ve	-ve
		Mannitol	-ve	-ve	+ve	+ve	+ve
4.	Citrate Utilization Test		+ve	+ve	+ve	+ve	+ve
5.	Casein hydrolysis test		+ve	+ve	+ve	+ve	+ve
6.	Catalase test		+ve	+ve	+ve	+ve	+ve
7.	MR/VP test		+ve/-ve	+ve/-ve	+ve/-ve	+ve/-ve	+ve/-ve
8.	Urease Test		+ve	+ve	+ve	+ve	+ve
9.	Starch hydrolysis test		+ve	+ve	+ve	+ve	+ve
10.	Indole test		-ve	-ve	+ve	-ve	-ve

(*R= Rod shaped ; +ve= Positive ; -ve= Negative)



*BS1-Bacterial species 1; BS2-Bacterial species 2;BS3-Bacterial species 3; BS4- Bacterial species 4. Figure 7: Effect of temperature on Alkaline Protease production by four different *Bacillus Spp.*

U/ml), BS2 (10.38 U/ml) and BS4 (9.41 U/ml) and lowest at 40°C as shown in Figure-7.

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