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Research Paper

Characterization of Bacillus Sp. nd Protease Production in Ssf

Microbiology

KEYWORDS : Protease, Bacillus sp., SSF, Characterization

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ABSTRACT

Different types of enzymes are produced by bacteria, but proteases are the most widely used. In the present study, protease producing Bacillus sp. was isolated from dairy effluent and charecterized. The isolate could use all carbohydrates tested and optimum pH, NaCl and temperature were 8.5, 2.25 and 37oC. Beef extract was most suitable carbon source. The isolate showed better growth in presence of Mg+2 and K+ salts but Fe+2 and Co+2 were most inhibitory. The isolate also produced amylase and CMCase but maximum yield was of protease after 48h in Smf, and in SSF 125 U protease/g biomass was produced in 1:9 proportion of cotton seed cake and rice husk.

Introduction:

Microbial proteases are among the three largest groups of industrial enzymes which secure approximately sixty percent of the total enzyme sale in the world (Rai and Mukharjee, 2010).

Proteases are produced and secreted into the medium only in presence of proteins. On the basis of fermentation condition, proteases differ each other in terms of stability, pH and temperature The need of the hour is isolating and charecterizing hyper-productive organisms growing on economical substrates for potential industrial applications. The economical feasible sources containing large amount of protein rich material that can be biologically transformed into recoverable products are obtained from dairy, meat and poultry processing industries (Raj et al., 2012). Organisms specifically selected on various substrates can give high yields of desirable enzymes. Media components, especially carbon, nitrogen sources and metal ions are the major factors which influence the extracellular protease production.

In the present study, a potential protease producing *Bacillus sp*. isolated from dairy waste contaminated soil was characterized.

Materials and method:

The protease producing culture was isolated from soil samples of local dairy. Its potentiality was selected by zone of hydrolysis on casein agar plate. Cultural and morphological charecteristics of the pure culture were studied after culturing on nutrient agar. Cultural characteristics like colony size, shape, elevation, texture etc., were studied. Confirmation of Gram character was done by Vancomycin test, KOH test and spore staining. The biochemical tests to study the metabolic charecteristics were performed as per the methods described in Bergey's Manual (Bergey's Manual of Systematic Bacteriology, 1984).

To study the effect of pH (2-10), temperature (10, 20, 30, 37 °C), NaCl (1-15 %), 100 mL nutrient broth was inoculated with 1 mL of 10^8cell/mL of actively growing culture and incubated at 37°C at 100 rpm and after 48 h the cell density was estimated using McFarland standards at 460 nm against their respective blanks.

Effect of various metal ions like $ZnCl_2$, CuCl, $CoCl_2$, $MgCl_2$, $MnCl_2$, EDTA, $MgSO_4$, K_2SO_4, $(NH_4)Fe(SO_4)$, $CuSO_4$, $ZnSO_4$, $FeSO_4$ were checked using 10 mM metal concentration.

Protease production in submerged fermentation was carried in medium containing the following (g/L): glucose 1.0, Casein 20.0, yeast extract 1.0, $CaCl_2$ 0.1, KH_2PO_4 0.5, $MgSO_4$ 0.1. The pH was adjusted to 8.5 before sterilization. Amylase and CMCase were produced in Nutrient broth containing 1% CMC and soluble starch respectively. For each enzyme production 100 mL sterile media was inoculated with 1 mL 0.3 x 10^8 cells/mL and incubated at 37°C, 2 mL of broth was aseptically removed after regular time interval and checked for enyme activity.

For protease production using SSF, Cotton seed cake and Rice husk were mixed in proportions of 9:1 7:3 1:1, 3:7, and 1:9 to make 10 g system in 100 mL conical flask. Moisture content was maintained 70% by adding above mentioned media without casein. The flask were sterilized and 5% w/w inoculum containing 10^8cell/mL of actively growing culture was added and incubated at 37°C. Fermented SSF boimass was removed after stipulated time and protease enzyme was extracted in 50 mL glycine buffer (8.5 pH). Protease assay was done by method decsribed by Kuberan *et al.,* (2010) protease activity was expressed in µMole of tyrosine released by 1 mL of enzyme in 30 minutes at 30 °C on tyrosine equivalent. Amylase and CMCase assay were done according to method given by Brinda *et al*., (2011) Amylase and CMCase was expressed in units (1 unit = amount of enzyme which releases 1 µMole glucose under the assay condition)

Results and discussion:

The cultural and morphological characteristics of the isolate

The colony of protease producing bacterial isolate isolate was intermediate in size with irregular shape and entire margin, the colony was slightly elevated with rough surface and creamish pigmentation. Morphologically isolate was long Gram positive rod, spore former occurred singly or in chains with positive motility, No gelling in KOH test and sensitive to Vancomycin. Protease is not single enzyme it include protinases, peptidases and amidases, among all mirobes and animals, proteases from *Bacillus sp*. are most significant (Liu *et al*, 1996; Ward, 1985). Oyeleke *et al.,* (2011) reported that protease enzyme produced by *Bacillus subtilis* showed superior activity compared to fungi.

Study of metabolic activities of the bacterial isolate

The isolate was able to utilize all the sugars used in this study as carbon source. The isolate also use citrate as a sole source of carbon. The isolate hasthe capability to hydrolyse casein, starch, and carboxy methyl cellulose. Details of biochemical characteristics exhibited by the isolate are shown in Table 1.

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Table 1: Results of biochemical tests

Positive Tests	Negative Tests
Glucose, Sucrose, Maltose, Lactose, Fructose, Mannose, Mannitol, Galactose, Arabinose, Raffinose, Trihalose, Cellobiose, Rhamnose, VP test, Citrate utilization, H_2S production, Starch hydrolysis, Casein hydrolysis, Gelatin hydrolysis, Growth at pH 5.7 and 6.8, Growth in 2, 5 and 7% NaCl, Growth at 20 and 30 and 37 °C.	Indole, MR, Nitrate reduction, Catalase, Growth in 10 % NaCl, Growth at 0 and 55 °C

Identification of the isolate

On the basis of cultural, morphological and metabolic charecteristics identification was done by ABIS online (http://www.tgw1916.net). Results indicated that the isolate has close similarity with *Bacillus tequilensis* and *Bacillus licheniformis*.

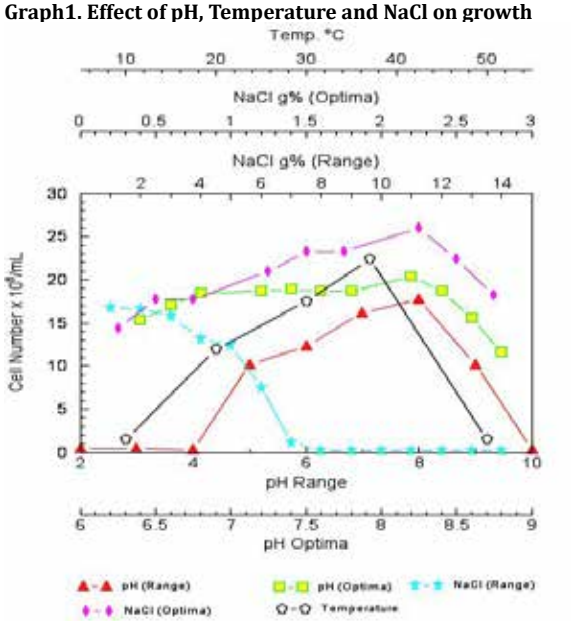
Effect of pH, Temperature and NaCl on growth

The *Bacillus sp*. grew over a pH range of 5.0 to 9.0 (Graph 1), the optimum pH was 8.4 here two fold increase in density was observed as compared to pH 5. At 9 pH difference was marginally less. It is well reported that growth and protease production is usually observed in alkaline pH *Bacillus Spp.* mostly produce proteases in two types ie., alkaline (8-10) and neutral (7-9) (Rao et al., 1998). Sharmin *et al*., (2005), isolated protease producing bacteria from degraded pulses and optimum pH for this isolate was 8.5. Aunstrup (1980) reported that the pH of the medium must be maintained above 7.5 in throughout the fermentation period of the protease production.

The isolated *Bacillus sp*. grew in media containing 1 to 6 %. NaCl, the optimal for growth was 2.25 % (Graph 1). The decrease in cell densities was 1.4, 2 and 16 fold at 3, 5 and 7% NaCl concentration respectively. Tarawneh *et al*., (2008) isolated seven halophilic bacterial strains that could utilize carbohydrate, casein and gelatin and divided them according to their NaCl tolerance in 3 groups tolerating 5 to 35 %, 1 to 15 % and 1 to 20 % NaCl respectively.

The isolated *Bacillus sp.* was grown in a temperatures range of 10°C to 50°C, the optimal was 37°C (Graph 1). At 30°C the cell density was 2 fold less compared to optimum. After 37°C abrupt fall in cell density was observed. On solid medium the isolate showed excellent casein hydrolysis at 37°C. Shafee *et al*., (2005), reported alkaline protease production from a newly isolated strain of *Bacillus cereus* that maximum protease at 37°C.

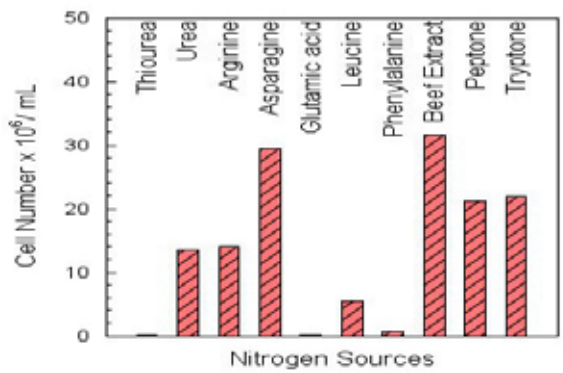
Graph1. Effect of pH, Temperature and NaCl on growth



Effect of Nitrogen sources on the growth of the isolate

The isolate was able to grow well on various nitrogen sources i.e., beef extract, asparagine, tryptone, peptone, arginine, urea and leucine (Graph 2). Maximum growth was obtained with beef extract followed by aspargine, peptone and tryptone. The isolate grew best at 0.75% Beef extract and further increase in its concentration had effect on growth.

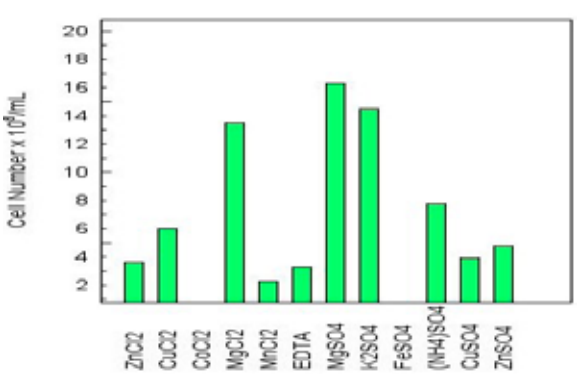
Graph 2. Effect of Nitrogen sources on the growth of the isolate



Effect of metals on the growth of the isolate

The bacterial isolate showed varied degrees of tolerance to the chloride and sulphate forms of metals at 10 mM concentration (Graph 3). The order of metal toxicity observed was $FeSO_4$ > $CoCl_2$> $MnCl_2$ > EDTA > $ZnCl_2$ > $CuSO_4$ > $ZnSO_4$ > $CuCl_2$>> NH_4SO_4 > K_2SO_4 $MgSO_4$. The maximum tolerance was observed with $MgSO_4$ and K_2SO_4 followed by $MgCl_2$, while $CoCl_2$ and $FeSO_4$ were most toxic to the isolate. Roy *et al.,* (2008) studying the effect of metals on the growth of protease producing bacteria showed that maximum tolerance was demonstrated for iron followed by lead and minimum tolerance was for metals like mercury, cadmium and silver.

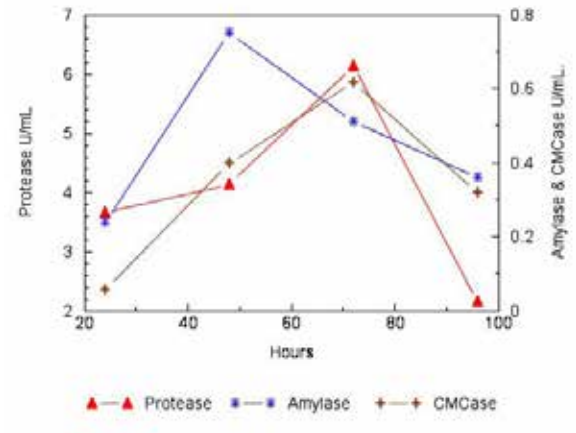
Graph 3. Effect of metals on the growth of the isolate



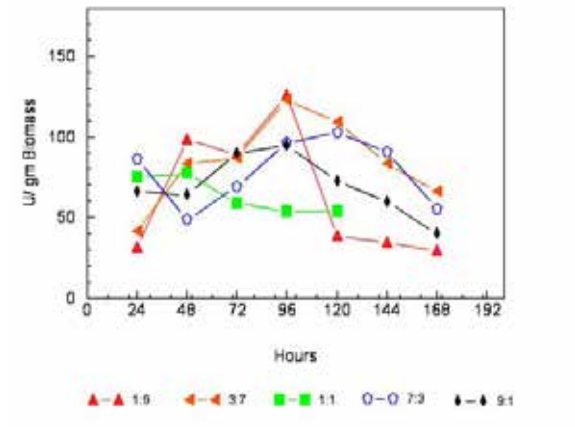
Production of hydrolytic enzymes by the selected isolate

The isolate is capable of producing various hydrolytic enzymes (Graph 4). Protease production was observed from 24 to 96 h. The maximum protease production was 6.13 U/mL after 72h., where as at 42 and 96 h the protease production was 4.14 and 1.1 U/mL respectively. The islolate also produced amylase (0.75 U/mL) and CMCase (0.64 U/mL) after 48 and 72 h respectively. Thankamani and Lipin, (2011) reported *Alcaligenes sp., Exiguobacterium sp., B. pumilus* and *B. fusiformis* producing extracellular alkaline proteases, amylases and cellulases *Alcaligenes sp.* produced high levels (37.38 Units) of amylase activity compared to 115 units of protease and 6.71 units of CMCase per mL of culture supernatant.

Graph 4. Production of hydrolytic enzymes by the selected isolate



wheat bran and defatted soybean meal using *Bacillus licheniformis.*



Protease Production by Solid State Fermentation:

Solid state fermentation for protease ptoduction was optimized with coton seed cake (nitrogen source) and rice husk in various proportions (Graph 5). Best combination of the biomas was 9:1. In this combination 125 U protease /g biomass was produced after 96 h. followed by 122.5, 77, 102 and 72 U/g biomass in proportions 3:7, 1:1, 7:3, 9:1 respectively. In recent years, solid substrate fermentation (SSF) has shown much promise in the development of bioprocesses and products of industry. Several reports on SSF have been published on the production of enzymes (Pandey, 2000: Suresh and Chandrasekaran, 1999), SSF is generally a simple process and requires less pre-processing energy than submerged fermentation. Furthermore, the initial capital costs are less compare with the SmF. Other advantages are; superior productivity, low waste water output and improved product recovery (Tunga *et al*., 1998). Nadeem (2008) reported alkaline protease production the medium containing

Conclusion:

The protease producing *Bacillus sp*., has good ability to ferment different types of sugars. Moreover, it hydrolysed starch, casein and carboxymethyl cellulose. The isolate grew well in a broad range of pH, NaCl and Temperature and the optima were 8.4. 2.25 % and 37°C respectively. The isolated *Bacillus sp*., utilized various simple and complex nitrogen sources and best growth was obtained in 0.75 %, beef extract. The isolate was able to produce multi enzymes i.e., protease amylase and CMCase. Protease production was higher compared to other two enzymes. In solid state fermentation 16 fold higher protease was produced compared to submerged fermentation. The isolate is a potential candidate for protease production using cheap agricultural biomass.

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