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Evaluation of Protease Production by a Halotolerant *Bacillus megaterium* (MTCC-9205) in Different Agro-Industrial Wastes Through Submerged Fermentation

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Abstract: In the present study, a well characterized and identified halotolerant bacterial strain (*Bacillus megaterium* MTCC-9205) was evaluated for its protease production. The bacterial strain when grown in skim milk agar plates showed the conspicuous zone of clearance around the colony indicating the protease production ability. Evaluation of extra cellular protease production by the bacterium was conformed in skim milk broth medium. Both acidic and alkaline protease production by the bacterium was screened in the skim milk broth medium using different substrates such as whey, soybean powder, black gram, green gram, wheat bran as cheap carbon sources. The highest yield of alkaline protease of 38.47 ± 1.32 U/ml by the strain was observed during the fermentation with soybean powder along with higher biomass (0.487±0.24 g/50 ml) production. Similarly acid protease was found to be 46.75 ± 1.22 U/ml with soybean as carbon source. The fermentation kinetics as well as yield factor of alkaline protease production was calculated and found maximum (12.95 U/g) in presence of soybean powder as carbon source. Further, the strain is immobilized and the activity of the enzyme was studied up to fourth cycle. The activity of the enzyme increased slightly with the use of recycled beads up to third cycle and fell thereafter. Hence, the present study revealed that the halotolerant *B.megaterium* is a potential protease producing bacteria which can be exploited for various industrial applications.

Keywords: *Bacillus megaterium,* protease, agro-industrial wastes.

Introduction

Microbial proteases are among the most important hydrolytic enzymes which have been studied extensively since the advent of enzymology. Due to its much commercial value, proteases dominate the world wide enzyme market, accounting for a two-third share of the detergent industry [26]. In the last 30 years, different classes of proteases with commercial importance have been produced from microbial, animal and plant sources which have implemented for enormous applications. Microorganisms represent an important source of proteases as they can be cultured in large quantities in a relatively short time by fermentation process which can produce large quantities of desired product. Besides, they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications [11, 20]. The vast diversity of proteases and the specificity of their actions have attracted worldwide attention to exploit their physiological and biotechnological applications. The extracellular proteases find multiple applications in various industrial sectors [10]. With increasing industrial demands for biocatalysts that can cope with the industrial processes at harsh conditions, the isolation and characterization of new promising strains are possible ways to increase the diversity and yield of such enzymes [9]. Currently, a large proportion of commercially available pro-

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teases are derived from *Bacillus* strain. Members of the genus *Bacillus*, from many different environments, have been explored and exploited for protease production. Some of the most protease producing bacilli strains are *Bacillus licheniformis*, *B. subtilis*, *B. amyloliquefaciens* and *B. mojavensis* including *B. megaterium* [22]. However, the protease productions from halotolerant *B. megaterium* isolated from saline habitats are less studied. Halotolerant proteases are important enzyme capable to withstand harsh conditions of salinity; temperature and pH and thus are required by a number of industries such as detergent formulation, tannery industries, therapeutic purposes and food industries [30].

It is well established that extracellular protease production in microorganisms is greatly influenced by media components, especially carbon and nitrogen sources, metal ions and physical factors such as pH, temperature, dissolved oxygen and incubation time [14]. At present, the overall cost of enzyme production is very high (due to high cost of substrate and medium used) and therefore, development of novel processes to increase the yield of proteases with decreasing the production cost is highly appreciable from the commercial point of view.

To achieve these goals, during the recent years, efforts have been directed to explore the means to reduce the protease production costs through improving the yield, and the use of low cost feed stocks or agricultural by products as substrates for protease production. Conventionally commercial production of protease has been carried out using sub-merged fermentation technology [2]. Modification of biotechnology and processes, using immobilized biocatalysts, has recently gained the attention of many biotechnologists. Application of immobilized enzymes or whole cells is advantageous because such biocatalysts display better operational stability and higher efficiency of catalysis and they are reusable [1]. The most common immobilization matrix used now a days is sodium alginate, since encapsulation in Ca-alginate gels occurs under very mild conditions and is characterized by the low cost [5].

In the present study, attempt has been made to screen protease production ability of a halotolerant *Bacillus megaterium* (MTCC-9205) species using different cheap agro-industrial wastes. Further, the optimization of the culture conditions and immobilized parameters for enhanced production of protease was also studied.

Methodology

Microorganism and culture maintenance

The bacterial strain used in this study was *Bacillus megaterium* MTCC-9205. The strain was isolated from soil samples of mangroves of Bhitarkanika, Odisha. Subsequently, the strain was identified using phenotypic and 16S rRNA gene sequencing at IMTECH, Chandigarh and deposited in the Microbial Type Culture Collection Centre, having accession number (MTCC-9205). Presently the strain is maintaining at Department of Biotechnology, MITS School of Biotechnology, Bhubaneswar. The strain was subcultured in nutrient agar medium at 37°C and used for the present study.

Screening of *B. megaterium* for protease production

The bacterial strain was spot inoculated on skim milk agar medium [23]. The inoculated plates were then incubated at 35°C. After this, the plates were flooded with $HgCl_2$-HCl solution for 5 minute. After 72 h of incubation, skim milk degradation was observed as a clearing zone around bacterial colonies. The clearing zone was measured, which indicates the extracellular protease activity of the bacterial strain.

Cultivation of *B. megaterium*

The strain was grown in 250 ml Erlenmeyer flask containing 50 ml of culture medium at 45°C for 48 h. The culture medium used for growing the strain contained (g/l) in distilled water (pH 7.0): casein, 10.0; glucose, 10; yeast extract, 3; peptone, 5; NaCl, 5; $CaCl_2.2H_2O$, 0.4 and $MgCl_2$ 0.2.

Agro-industrial residues for fermentation

Black gram peels (BGP), green gram peels (GGP), wheat bran (WB), whey, skimmed milk powder (SMP) and soybean powder (SP) were collected from the market of Bhubaneswar, Odisha, India. Each of these substrates was brought to the laboratory in new container. These agro-substrates were dried at 60°C for 48 h or

more until the moisture content was reduced. Cooled samples were later grounded in a blender and kept in sterile containers until required [29].

Fermentation

Liquid static surface fermentation for protease production

In 150 ml capacity Erlenmeyer flasks, 50 ml of sterilized fermentation medium and minimal salt solution ($ZnSO_4$ 62 mg/l; $FeSO_4.5H_2O$ 68 mg/l; $CuSO_4.5H_2O$ 8 mg/l; K_2HPO_4 1g/l) having individually black gram peels (BGP), green gram peels (GGP), wheat bran (WB), whey, skimmed milk powder (SMP) and soybean powder (SP) as substrate was inoculated with 1.0×10^7 cells ml^{-1} from 7 days old cultures of *Bacillus megaterium* were incubated at 30 ± 2°C at static condition [29]. After 96 h, samples were processed for recovery of protease and to study its activity.

Downstream processing of crude protease

After completion of fermentation, enzymes were extracted from the fermented media (LSSF) as per Sethi *et al*. method with slight modifications. In case of liquid static surface fermentation, harvested culture broth was cooled to 4°C for 1 hour after which each was centrifuged at 4 °C at 10,000 rpm for 30 min in an ultracentrifuge "Remi Compufuge CPR-24". The supernatant fluid obtained was thereafter used as crude protease for enzyme assay.

Separation and measurement of dry cell biomass

The biomass content was determined by measuring the dry weight from a known amount of sample. The sample was centrifuged at 10,000 rpm, at 4°C for 10 min and the bacterial biomass was rinsed with sterilized double distilled water and filtered out using Whatman No.1 filter paper. The biomass obtained was dried overnight inside the Hot Air Oven at 100°C till the constant weight was attained. Finally, the dry weight of the bacterial cell was weighed and calculated.

Immobilization of whole cells in sodium alginate

The alginate entrapment of cells was performed according to the method of Chatterjee [4]. Sodium alginate solution (3 %) was prepared by dissolving sodium alginate in 100 ml boiling water and autoclaved at 121°C for 15 minutes. Both alginate slurry and cell suspension were mixed well with a ratio of 1:1 for 10 minutes to get a uniform mixture. The slurry was taken into a sterile syringe and added drop wise into chilled 0.2 M $CaCl_2$ solution from 5 cm height and kept for curing at 4°C for 1 h. The beads were washed with sterile distilled water for 3 to 4 times. When the beads were not being used, they were preserved in 0.9 % sodium chloride solution in the refrigerator. All operations were carried out aseptically under laminar flow. Cells immobilized in beads of calcium alginate were inoculated in fermentation medium and incubated at 30°C.

Production of protease by repeated batch process

The reusability of immobilized *B. megaterium* cells in alginate was examined. Immobilized cells were aseptically inoculated to sterilized fermentation broth and incubated at 30°C for 96 h. Finally, the fermented media were filtered on Whatman No. 1 paper and beads were washed twice with sterile saline solution (0.8 % NaCl) and kept in phosphate buffer (1 M, pH 7.0). Then, the beads were again introduced into the fresh medium. After attaining maximum production of enzymes, the spent medium was replaced with fresh production medium (50 ml) and the process was repeated for five batches until the beads/ blocks started disintegrating. The enzyme titers and cell leakage of each cycle were determined.

Protease assay

Extracellular protease activity was determined according to the method of Van Den Hombergh *et al*., (1995). Each 450 µl sample was incubated with 50 µl 1 % (w/v) BSA in 0.1M sodium acetate buffer (pH 4.0) at 37°C. After 30 min of incubation, the reaction was terminated with 500 µl of 10 % (w/v) trichloroacetic acid (TCA). After incubation at 0°C for 30 min, the precipitated proteins were removed by centrifugation at 6000 rpm for 5 min, and the absorbance of the TCA-soluble fraction was estimated as per Lowry *et al*. method. One unit (U) of protease activity was defined as the amount of enzyme that releases 1

µg of tyrosine in 1 min under the assay conditions. Extracellular protease activity was expressed as U/ml. The specific activity of extracellular protease was defined as unit per gram of dry cell weight (U/g). Alkaline protease activity was measured following the standard method.

Fermentation kinetics study

Various fermentation kinetics parameters were also calculated. They include Ye/s (yield of enzyme per gram of substrate); Yx/s (yield of biomass per gram of substrate); Ye/x (yield of enzyme per gram of biomass); dx / dt {biomass accumulation in the culture medium (g l^{-1} h^{-1})}; d*P* D d*t* {enzyme accumulation in the culture medium (U ml^{-1} h^{-1}); μ_{max} {highest specific growth rate observed during batch culture ($mgl^{-1}h^{-1}$)}; x_{max} {maximum attainable biomass (mg ml^{-1})}; α {growth associated coefficient of enzyme production (U g^{-1})}; β {growth-independent coefficient of enzyme production (U g^{-1} h^{-1})}.

Results and discussion

Evaluation of growth and study of morphological characteristics

A strain of *B. megaterium* (MTCC 9205) previously isolated and identified from saline mangrove soil of Bhitarkanika, Odisha by the author was revived and used for the present experiment. The bacterial strain was aseptically streaked on Nutrient agar (NA) plates and the purity of the strain was checked under the microscope (Fig. 1). The bacter-ium was found to be Gram positive, motile, endo-spore forming rod. The bacterial growth was checked in NA medium containing 10 % NaCl (w/v). The strain was found positive for catalase test whereas negative for indole test. As previously studied, the strain had the ability to reduce nitrate while unable to hydrolyze gelatin and casein [18].

Fig. 1. Pure culture of *B. megaterium* on NA plates

Determination of protease activity and total protein content

The bacterium was grown in skim milk agar medium to determine its protease production ability. The strain MTCC-9205 produced transparent circular zones (halo zones) around the colonies in an opaque white background indicating protease production (Fig. 2). The hydrolyzing activity of the strain was measured and it was found 27 ± 0.24 mm after 72 h of incubation (Fig. 2). Similar type of study was undertaken by Ghasemia *et al*., [8] who screened two moderately halophilic bacteria for protease production using skim milk agar plate assay method. Since there is not necessarily good correlation between zones of clearing around colonies on skim-milk agar plates and levels of protease activity, the strain was further screened for protease production by assaying the protease activity in liquid culture using casein as substrate at 37°C and was found positive. The total protein content was quantified by Lowery method using BSA. The total protein content was found 578.45±3.31 µg/ml.

Fig. 2. screening of protease activity using *B. megaterium*

Quantitative screening of acidic and alkaline protease activity

For the production of protease, liquid static surface fermentation (LSF) was carried out using different substrates (whey, soybean powder, black gram peels, green gram peels, wheat bran, and skimmed milk powder) at 30°C up to 96 h. Production of both acidic and alkaline protease in LSSF was assessed. Results showed that whey and soybean powders as the substrates produced maximum acidic and alkaline protease. The bacterial strain could able to produce 48.45 ± 1.23 acidic and 37.25 ± 1.34 alkaline protease when grown in the presence of whey as carbon source. Whereas, in presence of soybean powder as a substrate the strain produced 46.75 ± 1.22 acidic and 38.47 ±1.32 alkaline protease. As compared to whey and soybean powders, other substrates had least effect on the production of alkaline protease (Fig. 3). During recent years, efforts have been directed to explore the means to improve the yield of protease production by use of either cost free or low cost feed stocks or agricultural by products as substrate like green gram husk which can reduce the protease production cost than that of other substrates [24]. The nature and amount of carbon source in fermentation media is important for extracellular protease production by bacteria. Cheaper source of carbon along with nitrogen sources are important for commercialization of enzyme production process. Akcan [2] has screened the production of extracellular protease in submerged fermentation by *B. lichiniformis* through supplement of different carbon sources such as soluble strach, wheat flour, rice flour, potato strach, corn strach, in the fermentation medium.

Fermentation study using free bacterial cell

Total protein and biomass production during fermentation

The total protein contents of the crude extracts were different when the fermentation was carried out with different substrates. Maximum protein content (578.45 ± 3.31 µg/ml) was found in the crude enzyme extract when the fermentation was carried out using skimmed milk powder (Fig. 4). *Bacillus megaterium* MTCC 9205 cells were aseptically inoculated to liquid static surface fermentation medium with different substrates and incubated for 96 h at 30°C. After successful fermentation, the biomass was separated, processed and estimated. The maximum biomass (dry weight of bacteria) of *Bacillus megaterium* MTCC 9205

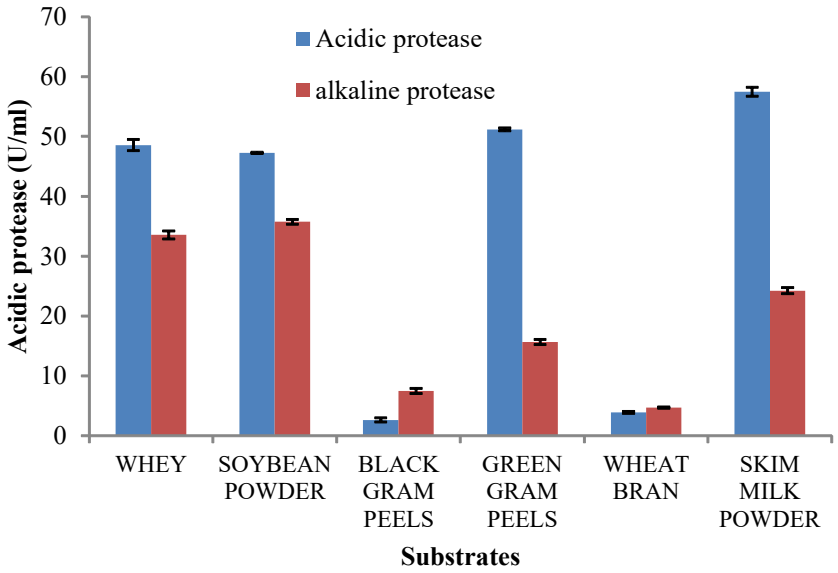

Fig. 3. Screening of different substrates for protease production through fermentation using *Bacillus megaterium MTCC 9205* at 30°C for 96 h incubation period (**WH**: whey; **SP**: soybean powder; **BGP**: black gram peels; **GGP**: green gram peels; **WB**: wheat bran; **SMP**: skimmed milk powder)

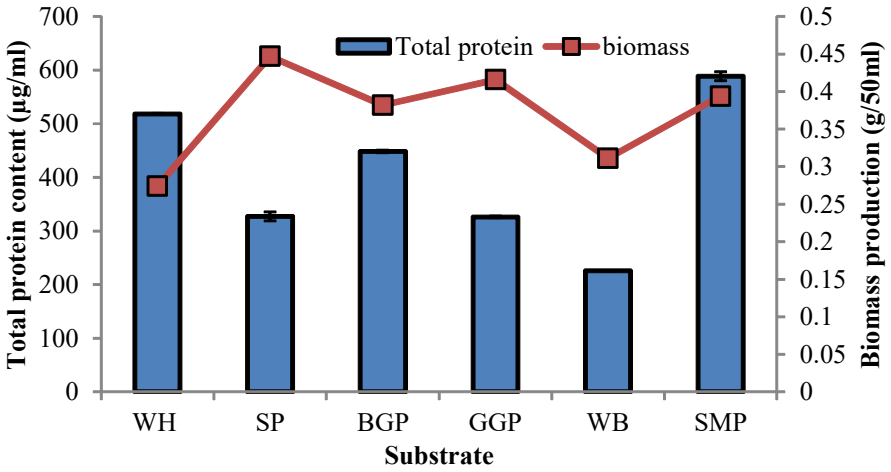

Fig. 4. Total protein content and biomass produced by *Bacillus megaterium MTCC 9205* under different agro-residues using liquid static surface fermentation carried out at 30°C for 96 h of incubation

was obtained with soybean powder (0.487 ± 0.24 g/50ml) as compared to other substrates taken for this study (Fig. 4).

Several investigations have been done for screening of new isolates for protease production. Studies reported that *Bacillus anthracis* S-44 and *Bacillus cereus* S-98 exhibited their maximum ability to biosynthesize proteases within 60 hours of incubation period and the productivity reached up to 126.09 units/ml for *Bacillus anthracis* S-44 and 240.45 units/ml for *Bacillus cereus* S-98 [13]. Several investigations done in alkaline protease enzyme production by *Bacillus* sp. has been previously reported [7, 28].

Alkaline protease assay and determination of specific activity

As alkaline protease is an important enzyme, attempt has been made to estimate its activity (U/ml) as well as its specific activity (U/mg). The specific activity of the enzyme (AP) was estimated from the obtained data and it was concluded that maximum specific activity of the alkaline protease (117.64 U/mg proteins) produced using soybean powder as the sole source of carbon (Table 1). So far, no defined medium has been established for the optimum production of alkaline proteases from different microbial sources. The study made by Mariana *et al.*, [17], revealed that soybean extract and soybean flour were good inducers for alkaline protease production in submerged fermentation (SmF).

Fermentation kinetics

The fermentation kinetics parameters were calculated after the successful completion of fermentation. The various fermentation yield factors were calculated and presented in the Table 1. It was concluded from the table that the highest yield of protease by *Bacillus megaterium MTCC 9205* was observed during the fermentation was with soybean powder i.e. 12.95 U/g (Table 2). Simi-

Table 1. Specific activity of protease produced by *Bacillus megaterium MTCC9205*

Bacteria	Substrates	Protease activity (U/ml)	Total protein (mg/ml)	SA (U/mg)
Bacillus megaterium MTCC-9205	Whey	37.25 ± 1.34	0.519	71.77
	Soybean powder	38.47 ± 1.32	0.327	117.64
	Black gram peels	8.67 ± 1.25	0.448	19.35
	Green gram peels	14.46 ± 2.15	0.326	44.35
	Wheat bran	4.22 ± 0.28	0.226	19.18
	Skimmed milk powder	24.64 ± 0.47	0.589	41.83

Table 2. Fermentation kinetics of the protease produced by *Bacillus megaterium MTCC 9205*

Substrates	$Y_{e/s}$ (U/g)	$Y_{x/s}$ (g/g)	μ (h-1)	β (U/g/h)	d*P*/d*t*(U/ml/h)
Whey	11.15	0.05	0.002	0.116	0.548
Soybean powder	12.95	0.08	0.004	0.134	0.670
Black gram peels	5.59	0.07	0.003	0.058	0.291
Green gram peels	2.28	0.08	0.004	0.023	0.111
Wheat bran	2.56	0.06	0.003	0.026	0.130
Skimmed milk powder	11.17	0.07	0.004	0.116	0.581

larly, the protease accumulation in the culture medium per hour (dP/dt) was highest when soybean powder was used as the substrate. But, the growth-independent coefficient of enzyme production (β) was optimum when fermentation was carried out by *Bacillus megaterium* using soybean powder followed by whey and skimmed milk powder, respectively. The fermentation kinetics parameters were calculated after the successful completion of fermentation. The various fermentation yield factors were calculated and it was concluded from the data that the highest yield of protease (12.95 U/g) by *B.megaterium* was observed during the fermentation was with soybean powder Similarly, the protease accumulation in the culture medium per hour (dP/dt) was highest when soybean powder was used as the substrate.

Fermentation using immobilized cells of *B. megaterium*

The cell of *Bacillus megaterium* MTCC 9205 was immobilized using sodium alginate for the production of protease and its reusability was evaluated. Immobilized cells of the bacterium were aseptically inoculated to fermentation flasks in the presence of soya bean powder as the carbon sources. The activity of protease increased slightly with the use of recycled beads up to third cycle and fell thereafter when soybean powder was used as the carbon source. The production of protease was noticed at every cycle and continued up to 4th cycle but the activity slowed down after 3rd cycle. A peak in enzyme activity was observed at 2nd cycle by the immobilized cells of *Bacillus megaterium* MTCC 9205 (Fig. 5).

Immobilization of bacterial spores/cells and their enzymes has become one of the most precious techniques in the field of modern biotechnology [6]. The encapsulation provides prolonged metabolic activity when microbial cells are repeatedly used and it also provides protection to the organism from inhibitory compounds or metabolites. Similar type of study was made by Adinarayana *et al*. [1]. When immobilized the bacterial cells in calcium alginate and found more production of alkaline protease with repeated batch fermentation. The calcium alginate immobilized cells of *B. subtilis* PE-11 can be proposed as an effective biocatalyst for repeated usage for maximum production of alkaline protease [19]. By immobilization of microbes in different entrapment matrices, the enzyme produced can be more stable, pure, continuous and can be reused which in turn modulates the enzyme production in an economical manner. There have been reports in support of calcium alginate as excellent matrices for immobilization of *B. subtilis* and *B. licheniformis* for protease production [4].

In the world market half of the enzymes are produced by *Bacillus* sp. and approximately 60 % of these enzymes are proteases [27]. Over 300 tones of enzymes, mainly proteases are annually produced from *Bacillus* sp. [16]. Of the total enzymes used in various commercial and industrial processes are obtained from mesophilic microrganims which are inefficient for their harsh industrial conditions. On the contest, enzymes from halotolerant bacteria are useful in many harsh industrial processes where concentrated salt solutions are used [12]. Halotolerant alkaline proteases exhibit high proteolytic activity and stability under alkaline and high saline environment. Thus, these enzymes have extensive applications in industries

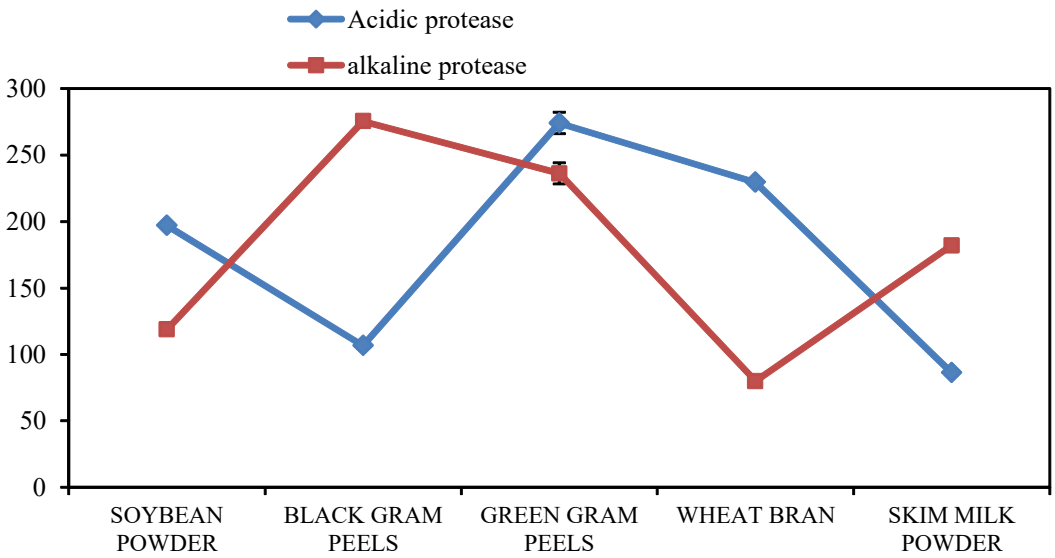

Fig. 5. Repeated batch culture using calcium alginate immobilized cells of *Bacillus megaterium MTCC 9205* (Second cycle)

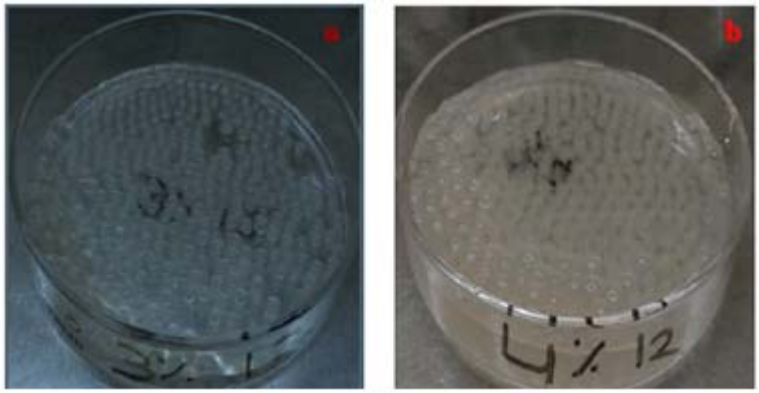

Fig. 6. Immobilized cells of *Bacillus megaterium*

like laundry detergents, pharmaceutical, food, leather and feather processing and proteinaceous waste bioremediation [32]. Researchers have been focused in protease production from halotolerant bacterial species [30, 31]. The present study is another attempt in this direction. Isolation of protease from halotolerant *B. megaterium* could be a potential enzyme for many industrial applications. However, further study is required for industrial production and its applications in various sectors.

Conclusion

The present study deals with the screening of different agro-industrial wastes for the production of protease. Maximum alkaline protease and bacterial biomass was produced when *B. megaterium* was inoculated in fermentation medium containing soya powder as cheap carbon source. During immobilization study, it has been observed that in 4 % (w/v) sodium alginate beads entrapping *B. megaterium* cells, the production of protease continued with increase in production up to 4th cycle. The results of this work revealed that the halotolerant *B. megaterium* species could display a potential role in protease production both in the free and immobilized condition using cheap carbon sources such as whey and soybean powder which can be exploited for many biotechnological applications. Due to importance of this finding further optimization of process parameters are necessary for enhanced enzyme production.

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