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ORIGINAL PAPER

Enhanced production of alkaline thermostable keratinolytic protease from calcium alginate immobilized cells of thermoalkalophilic *Bacillus halodurans* JB 99 exhibiting dehairing activity

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Abstract The thermoalkalophilic *Bacillus halodurans* JB 99 cells known for production of novel thermostable alkaline keratinolytic protease were immobilized in calcium alginate matrix. Batch and repeated batch cultivation using calcium alginate immobilized cells were studied for alkaline protease production in submerged fermentation. Immobilized cells with 2.5% alginate and 350 beads/flask of initial cell loading showed enhanced production of alkaline protease by 23.2% (5,275 ± 39.4 U/ml) as compared to free cells (4,280 ± 35.4 U/ml) after 24 h. In the semicontinuous mode of cultivation, immobilized cells under optimized conditions produced an appreciable level of alkaline protease in up to nine cycles and reached a maximal value of 5,975 U/ml after the seventh cycle. The enzyme produced from immobilized cells efficiently degraded chicken feathers in the presence of a reducing agent which can help the poultry industry in the management of keratin-rich waste and obtaining value-added products.

Keywords Calcium alginate · Immobilization · Repeated batch cultivation · Thermostable alkaline protease · *Bacillus halodurans* JB 99

Introduction

Microbial alkaline proteases (E.C.3.4.21) are important constituents of commercial enzymes which account for more than 60% of the total industrial enzyme market. Alkaline proteases produced from thermophilic and alkalophilic *Bacillus* which can withstand high temperature, pH, chemical denaturing agents and non-aqueous environments have attracted a great deal of attention due to their multitude of industrial applications such as in the detergent, leather, pharmaceutical (preparation of ointments) and fertilizer industries and also in biotechnological applications such as peptide synthesis, management of waste from various food-processing industries, silver recovery from X-ray or photographic films and proteinaceous fodder from waste feathers or keratin-containing materials [7, 10, 11, 22]. Owing to these properties, protease exhibiting keratinolytic activity also plays a significant role in combating environmental pollution caused by chicken feathers generated from the poultry industry. The conversion of feathers to feather meal (which is used as supplement in animal feed) requires high energy input, but instead keratinolytic protease can be used in a biotechnological process to upgrade the high nutritional value of feather meals [9, 18]. The industrial applications have been limited by several factors such as the high cost of the enzymes, their instability at high pH and temperature, and their availability in small amounts [5, 12, 13]. Among many biotechnological approaches, immobilization of microbial cells as biocatalyst has recently gained the attention of many biotechnologists, because of its advantage over conventional free cell systems in respect to operational stability, higher efficiency of catalysis, higher volumetric productivity, reusability and retention of high cell density [2, 6, 12]. The most widely used method for immobilization of whole cells in biotechnology at a laboratory scale, and also in some cases industrial scale, is entrapment. Many gel-like materials are used as carriers which may be based on natural (alginate, *k*-carrageenan, agar, chitosan etc.) or synthetic (polyacrylamide, polyurethane etc.) substrates. The entrapment of microbial cells in

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alginate by ionotropic gelation using divalent cations has found extensive use in immobilized viable cells technology, because of is nontoxic in nature, inexpensive and simple to perform. The entrapment of cells can be achieved under mild conditions without affecting the cell viability and growth [1, 6]. Immobilization of viable cells using alginate as entrapment matrix has been extensively used for production of a wide number of biomolecules (antibiotics, enzymes, organic acids) and secondary metabolites (steroids, alcohol etc.) and also for degradation of wastewater pollutants [4–6, 8, 13, 16, 21]. There are very few reports which describe thermostable alkaline protease production by Ca-alginate immobilized cells exhibiting keratinolytic activity [1, 2, 25]. Our laboratory has isolated and biochemically and molecularly characterized a novel *Bacillus halodurans* JB 99 strain having industrially important characteristics like thermal stability, pH stability and high enzyme yield. We have previously reported the production and characterization of thermostable alkaline keratinolytic protease from thermoalkalophilic *Bacillus halodurans* JB 99 using pigeon pea waste as a novel substrate with broad stability at pH 10.0–11.0, temperature 60–80°C, in the presence of detergents and metal ions, and exhibiting dehairing activity [15, 23]. As a result of the increasing demand for enzymes in the leather industry, management of feather waste and several other applications, the present investigation was carried out to optimize conditions required for enhancement of alkaline keratinolytic protease production from calcium alginate immobilized cells of *Bacillus halodurans* JB 99 using batch and semicontinuous modes of cultivation and its application in the degradation of chicken feathers.

Materials and methods

Bacterial strain, maintenance and molecular identification

The thermoalkalophilic *Bacillus halodurans* JB 99 used in the present investigation was isolated in our laboratory from sugarcane molasses and biochemically characterized at the Institute of Microbial Technology (IMTECH), Chandigarh, India. The culture maintenance procedure and characterization were carried out as described previously [23, 25]. The molecular identification of the strain was confirmed by 16S rRNA gene sequence analysis. The 16S rRNA gene was sequenced at the National Centre for Cell Science (NCCS), Pune, India. The DNA sequence was screened against the National Centre for Biotechnology Information (NCBI) GenBank database using the BLAST program and it was submitted to the database with the accession number DQ406675.

Preparation of inoculum

The 18-h-old culture of *Bacillus halodurans* JB 99 (1.5×10^7 CFU/ml) was inoculated into 250-ml Erlenmeyer flasks containing 50 ml of sterile inoculum medium at the 5% level. The composition of the inoculum medium used was (g/l) casein hydrolysate, 1.0; yeast extract, 2.5; peptone, 5.0; $NaNO_3$, 10.0; KH_2PO_4, 5.0; $MgSO_4 \cdot 7H_2O$, 0.3; $CaCl_2$ 0.2, NaCl, 5.0; sodium carbonate, 10.0; pH 10.5. The flasks were incubated on a rotary shaker at 220 rpm at 45°C. The contents of the flasks were centrifuged at 3,000 rpm for 10 min and the supernatant was decanted. The cell pellet was washed thoroughly with sterile sodium chloride solution (9.0 g/l) and then sterile distilled water. Finally, the cell mass was suspended in sterile sodium chloride solution (9.0 g/l). This cell suspension (adjusted to a cell density of 2.0×10^9 CFU/ml) was used for immobilization and free cell fermentations. Equal cell densities (2.0×10^9 CFU/ml or CFU/g) were maintained for free and immobilized experiments.

Immobilization of whole cells in calcium alginate

The gel matrix was prepared by dissolving the sodium alginate (3% w/v) in boiling water with continuous stirring to avoid undissolved lumps and sterilized by autoclaving at 121°C for 15 min. The cell suspension and alginate slurry were mixed and stirred continuously for 10 min to obtain a uniform mixture with final concentration of 2.5% alginate. To this, glutaraldehyde (2.5% w/v) was added to give a final concentration of 0.1% (w/v), gently mixed and then kept for 90 min. The homogenous mixture was added dropwise into cold sterilized 0.25 M $CaCl_2$ solution with the help of a sterile syringe from a height of 6 cm to obtain beads of uniform size ($\sim$3.0 mm) and kept for curing for 1 h at 4°C in fresh 0.25 M $CaCl_2$. The cured beads were washed 3–4 times with sterile distilled water. The beads having cell density of 2.0×10^9 CFU/g were then utilized for batch fermentation in 250-ml Erlenmeyer flasks containing 50 ml of culture media (g/l) casein hydrolysate,1.0; yeast extract, 2.5; peptone, 5.0; $NaNO_3$, 10.0; KH_2PO_4, 5.0 $MgSO_4 \cdot 7H_2O$, 0.3; $CaCl_2$ 0.2, NaCl, 5.0; pH 9.5. Cultivation was carried out on an orbital shaker at 120 rpm at 37°C. All operations were carried out aseptically.

Enzyme assays

Keratinolytic and caseinolytic activities were determined by using keratin and casein as substrates [17]. A total of 2 ml of reaction mixture containing 1 ml of casein 1% (w/v) and 0.9 ml of 50 mM glycine/NaOH buffer (pH 11.0) was preincubated at 70°C. Reaction was initiated by the addition of 100 µl of suitably diluted enzyme solution

and kept at 70°C. The reaction was terminated after 20 min by adding 2 ml of 10% trichloroacetic acid (TCA). The reaction mixture was centrifuged at 12,000 × *g* for 10 min and absorbance (*A*) was measured at 280 nm. Keratinolytic activity was studied using 1% keratin (Himedia, India) as a substrate in place of casein in the above reaction mixture. One unit of caseinolytic and keratinolytic activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per minute per millilitre under the experimental conditions.

Effect of alginate concentration and initial cell loading (ICL) on protease production

The effect of different concentrations of alginate and ICL on protease production was determined. To determine the optimum concentration of sodium alginate for cell immobilization, various concentrations of sodium alginate (1, 2, 2.5, 3 and 4% w/v) were used to prepare the beads, and cell loading in terms of bead numbers from 100 to 400 were used for inoculating fermentation media. The beads were prepared as described earlier in 0.25 M $CaCl_2$ solution, transferred into the production medium and incubated at 37°C for 24 h.

Enumeration of entrapped cells and cell leakage

The content of the cells entrapped in the alginate beads was measured by dissolving the gel beads in sodium pyrophosphate (1% w/v) followed by gentle homogenization. The bacterial count in this solution and cell leakage from the beads during course of fermentation were measured after serial dilution and by plating on nutrient agar plates (pH 10.0) by incubation at 45°C.

Repeated batch cultivation

In the repeated batch process, after attaining the maximum production of alkaline protease (24 h) the fermented medium was replaced with fresh sterilized production medium (as described above) and the process was repeated for nine cycles of fermentation of 24 h. The enzyme activity and cell leakage of each cycle were determined.

Feather degradation activity of protease

Feather disintegration was studied using ethanol-washed chicken feathers which were incubated with crude enzyme preparation supplemented with and without 0.1% v/v β-mercaptoethanol (β-ME) at 45°C for 24 h on a rotary shaker at 120 rpm. The level of degradation was assessed by periodic observation of the enzyme-treated feathers.

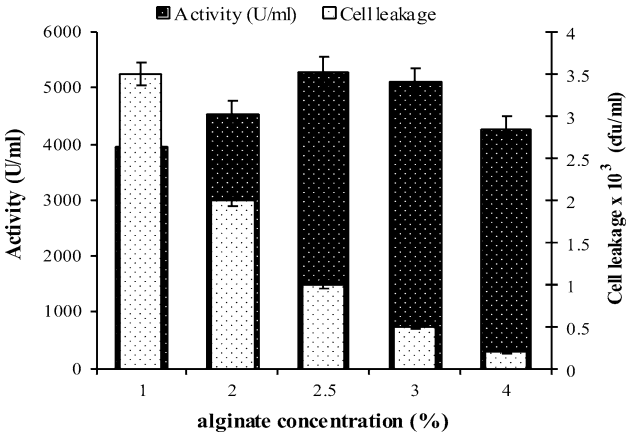

Fig. 1 Effect of alginate concentration on alkaline protease production by immobilized *Bacillus halodurans* JB 99

Results

Effect of alginate concentration

Different concentrations of alginate (1.0, 2.0, 2.5, 3.0 and 4.0%) for immobilization of cells were used for evaluation of the maximum alkaline protease production. The results are depicted in Fig. 1. Alkaline protease production increases with increasing alginate concentration from 1 to 2.5% and reached a maximal value at 2.5%. Initially, at low concentration of alginate, there was less enzyme production with acute cell leakage; at increased alginate (>2.5%) concentration a decline in protease production and cell leakage was observed as a result of reduced porosity of the beads limiting the nutrient supply and oxygen diffusion.

Effect of ICL

The ICL was tested by varying bead numbers from 100–400 in each flask using 2.5% alginate concentration. Alkaline protease production increased as a function of the number of beads and maximal production was reached with 350 beads per flask (Fig. 2).

Comparison of thermostable alkaline protease production by immobilized and free cells of *Bacillus halodurans* JB 99

Enzyme production by free and immobilized cells was studied. In the case of free cells, maximum alkaline protease production (5,065 ± 37.8 U/ml) was obtained after 40 h of growth, whereas in the case of alginate-entrapped cells it reached a maximum level of 5,275 ± 39.4 U/ml after 24 h with comparatively higher activity (Fig. 3a, b).

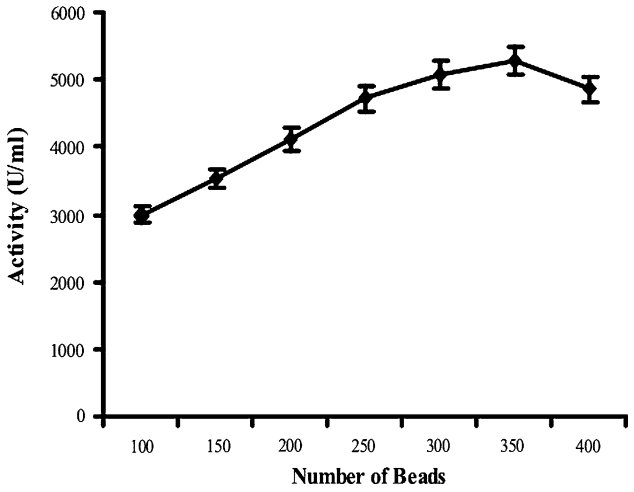

Fig. 2 Effect of ICL on alkaline protease production by immobilized *Bacillus halodurans* JB 99

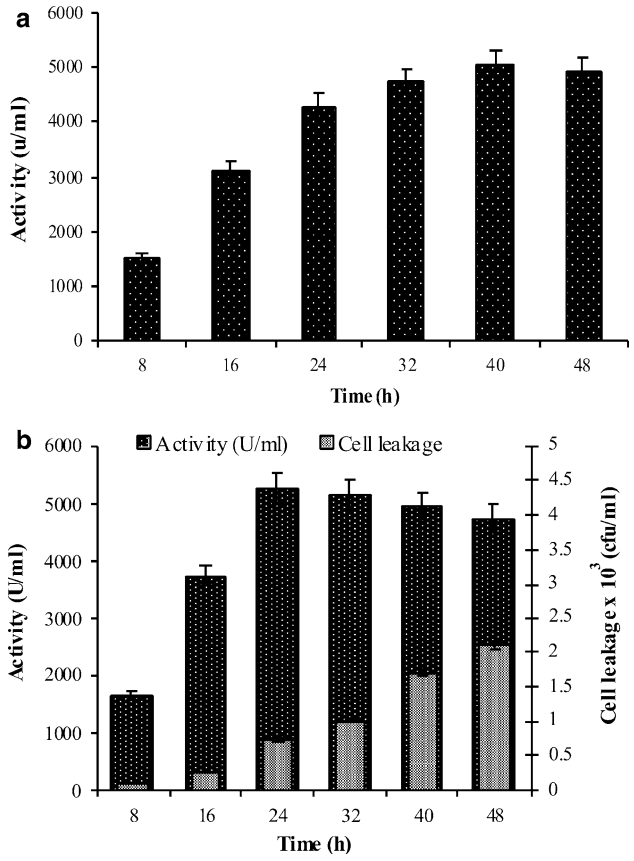

Fig. 3 **a** Time course profile of alkaline protease production by free cells of *Bacillus halodurans* JB 99; cultivation was done at 37°C for 48 h on an orbital shaker at 120 rpm. **b** Time course profile of alkaline protease production and cell leakage (cfu/ml) by immobilized *Bacillus halodurans* JB 99; cultivation was done at 37°C for 48 h on an orbital shaker at 120 rpm

The alkaline protease production in the immobilized system was enhanced by 23.2% as compared to the free cells system at the same time interval (24 h).

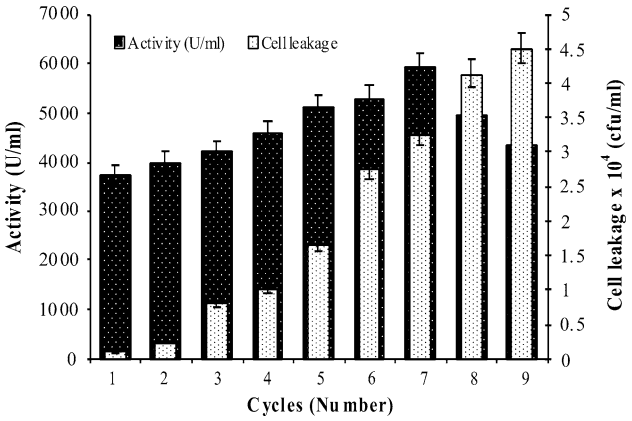

Fig. 4 Repeated batch production of alkaline protease by *Bacillus halodurans* JB 99 cells immobilized in calcium alginate. Each cycle was carried out for 24 h in a rotary shaker at 120 rpm

Reuse of the immobilized cells for repeated batch cultivation

The semicontinuous mode of cultivation was carried out by immobilized cells with 2.5% alginate concentration and ICL of 350 beads in order to determine the stability of beads and alkaline protease production under repeated batch growing conditions. The cultivation was continued for nine consecutive 24-h cycles. There was a linear increase in production of thermostable alkaline protease and the production peak was attained at the seventh cycle (5,975 ± 32.5 U/ml) (Fig. 4). Cell leakage was also determined during the course of fermentation.

Feather disintegration

The level of disintegration was visually observed at various times during a 24-h incubation. Disintegration of side branches (barbs) started after 8 h (data not shown) of enzymatic treatment with 0.1% β-ME and without β-ME and degradation up to 85 and 70%, respectively, was observed after 24 h of incubation leaving behind the thick basal shaft as shown in Fig. 5. The influence of β-ME on reducing the disulphide bond in keratin protein of feathers increased the rate of disintegration as compared to treatment without 0.1% β-ME.

Discussion

Whole cell immobilization has several advantages in microbial fermentations because of its high efficiency and low production cost. These cells are more stable, active and reusable; and can eliminate most of the constraints faced by free cells. Several examples of production of a variety of biomolecules by immobilized cells have been successfully

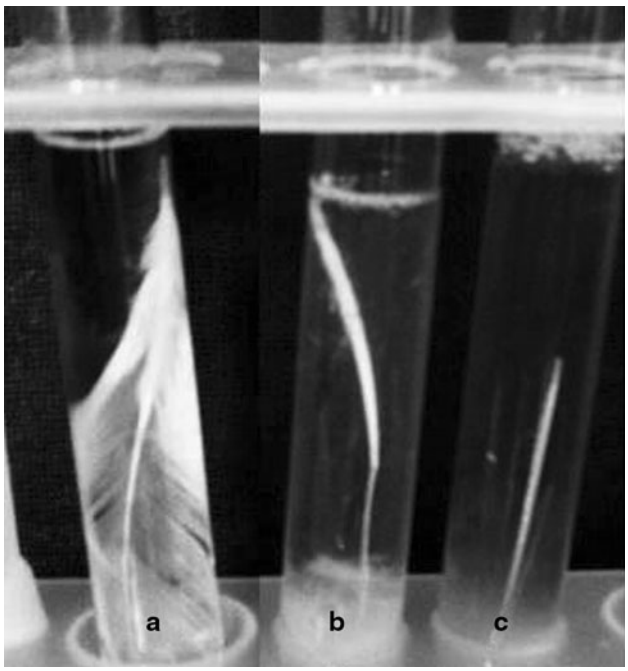

Fig. 5 Disintegration of native chicken feathers by crude enzyme preparation (**a** control; **b** crude enzyme without β-mercaptoethanol; **c** crude enzyme with β-mercaptoethanol) of *Bacillus halodurans* JB 99 at 45°C for 24 h on a rotary shaker at 120 rpm

demonstrated [6, 20]. Microbial keratinolytic proteases have been described for various biotechnological applications in the food and leather industries, yet the growing demand for these enzymes necessitates the screening for novel keratinolytic microorganisms [10].

The aim of the present study was to investigate the thermostable alkaline keratinolytic protease production and stability of alginate-entrapped cells of *Bacillus halodurans* JB 99 in the semicontinuous mode of cultivation. The thermostable, alkaline and keratinolytic nature of protease from *Bacillus halodurans* JB 99 makes its suitable for use in the detergent, leather and poultry industries and also in bleaching agents because of its stability in the presence of surfactants [15, 23]. Enzyme production by immobilized cells depends upon several factors such as alginate concentration, cell load and number of cycles. In the present investigation, alginate concentration and amount of cells were optimized to produce enhanced levels of alkaline protease from immobilized cells as compared to free cells. Hardly any reports have been published on the production of thermostable alkaline protease from thermoalkalophilic *Bacillus* sp.

The influence of alginate concentration on production of alkaline protease was investigated to evaluate the stability of calcium alginate beads; various concentrations of alginate (1, 2, 2.5, 3 and 4) were used for the immobilization of *Bacillus halodurans* JB 99. Alginate at 2.5% was found to be the optimum concentration for maximum alkaline protease production with moderate cell leakage. Adinarayan et al. [1] reported 3% alginate concentration as optimal for protease production from *Bacillus subtilis* PE-11 and Subba Rao et al. [24] also reported 2.5% alginate concentration for alkaline protease production from *Bacillus circulans* (MTCC6811). The effect of ICL (in the form of the number of alginate beads per flask) on alkaline protease production was studied. The alkaline protease production increased for ICLs up to 350 beads per flask and with increased stability. A similar observation was reported by Kar et al. [16] who used 300 beads for xylanase production from *Trichoderma reesei* SAF3; Beshay [3] also reported 300 beads for maximum alkaline protease production from *Teredinobacter turnirae*; and Pradeep et al. [19] showed maximum production of thermostable and neutral glycoamylase with 40 beads per 50 ml production medium using Ca-alginate immobilized *Thermomucor indicae-seudaticae*. During the present work, freely growing and immobilized cells followed similar fermentation profiles during batch cultivation. Higher activity was noticed after 24 h for free cells compared with 40 h for the immobilized cells system. This indicates good stability of entrapped active and induced cells during enzyme production and requires less time. On the other hand, free cells required more time for induction of enzyme synthesis and attainment of a certain cell mass.

One of the important features of Ca-alginate–cell complexes is the longevity of biochemical activities, which will have a significant economic advantage. Under such conditions the risk of contamination will be low, thus the cost of downstream processing will be reduced [8, 9]. In repeated batch cultivation, entrapped *Bacillus halodurans* JB 99 cells exhibited better stability in terms of enzyme activity and production in up to seven cycles of fermentation with minimal cell leakage. After seven cycles, the decrease in production was associated with increased cell leakage from the beads. Earlier investigators reported similar observations regarding protease productivity and activity. Vuillemard et al. [26] reported protease production by immobilized *Serratia marcescens* and *Myxococcus xanthus* in calcium alginate beads, which reached a maximum after five cycles (5 days) for *S. marcescens*. Adinarayan et al. [2] reported similar findings with *Bacillus subtilis*, which retained its ability to produce alkaline protease for 9 days. Jamuna et al. [14] also reported continuous synthesis of thermostable amylase from *Bacillus* cells immobilized in calcium alginate beads for 10 days. Based on these findings, further scale-up experiments can be carried out with calcium alginate immobilized thermoalkalophilic *Bacillus halodurans* JB 99 cells, which should provide useful data for large-scale production of alkaline protease with enhanced productivity.

To conclude, the results showed that Ca-alginate entrapment may be a promising method for immobilization of *Bacillus halodurans* JB 99 for alkaline keratinolytic protease production. Alginate is a nontoxic product and, therefore, immobilized bacterial cells could be useful for large-scale production of keratinolytic protease. It is significant that there are very few reports on immobilization of thermoalkalophilic *Bacillus* sp. for keratinolytic protease production. The enzyme could also able to hydrolyze keratin-rich waste from the poultry industry and afford value-added products through an energy-saving and eco-friendly approach.

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