PROTEASE PRODUCTION BY IMMOBILIZED CELLS OF TEREDIOBACTER TURNIRAE USING VARIOUS MATRICES ENTRAPMENT TECHNIQUE

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Abstract

An attempt was made to study the protease production by immobilized cells of *Terediobacter turnirae* using different matrices such as calcium alginate, k- carrageenan, polyacrylamide, agar-agar and gelatin gel. Among the different matrices calcium alginate was found to be better than other matrices for higher protease production. Influence of different pH and temperature on production was also studied. The maximum production was found at pH 8 and temperature 60 0C. In the repeated batch fermentation, calcium alginate was more efficient for the production of protease than other matrices.

Keywords: Batch fermentation, Immobilized cells, matrices, calcium alginate, *Terediobacter turnirae*

I. INTRODUCTION

Proteases form a large group of enzymes, ubiquitous in nature and found in a wide variety of microorganisms. Proteases constitute one of the most important groups of industrial enzyme that are now used in a wide range of industrial processes such as detergent, food, pharmaceutical, leather and silk industries (Prakasham et al., 2005; Ellaiah P et al., 2003 and Adinarayanan & Ellaiah P. 2003).

Among the various proteases, bacterial proteases are the most significant compared with animal and fungal proteases. Amonge bacteria, Bacillus species are specific producers of extracellular proteases. These proteases have wide applications in various industries (Pastor et al., 2001; Ward, 1985). In detergent industry, it has emerged as one of the major consumers of hydrolytic enzymes working at alkaline pH, and now accounts for more than one quarter of the global enzyme production (Adinarayanan et al., 2003). At present, the use of alkaline protease has increased remarkably which are derived from Bacillus strains (Adinarayanan & Ellaiah P, 2004; Sen & Satyanarayana, 1993).

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 (Fortin & Vuillemard, 1990) and higher efficiency of catalysis (Ramakrishna et al., 1992) and they are reusable.

Microbial products are usually produced either by free or immobilized cells. The use of immobilized cells are industrial catalysts can be advantageous compared to batch fermentation processes (Venkatasubramanian, 1979). Whole cell immobilization has been a better choice over enzyme immobilization (Ramkrishna & Prakasham,1999).

Whole cell immobilization by entrapment is a widely used and simple technique. Romo and Perezmartinez (1997) reported the viability of microbial cells over a period of 18 months under entrapped conditions and it was considered as one of the potential application. The success achieved with the entrapment technique prompted us to study the production of alkaline protease with immobilized cells using this technique.

The present investigation was to study the immobilization of Terediobacter turnirae cells for higher protease production using different entrapment techniques with matrices such as calcium alginate, k-carrageenan, polyacrylamide, agar-agar and gelatin gel. The reusability of immobilized cells for protease production under repeated batch fermentation was also investigated.

II. MATERIAL AND METHODS BACTERIAL STRAIN

An alkaline protease producing strain of Terediobacter turnirae was isolated using milk agar as a substrate (i.e., Skimmed milk powder 0.1% and 0.2%) and maintained on nutrient agar slants at 4oC. Subculture was carried out for every 4 weeks.

Inoculum Preparation

5 ml of sterile distilled water was added to a 24 hrs old slant of Terediobacter turnirae The cells were scrapped from the slant into sterile distilled water and the resulted cell suspension at 10% level was transferred aseptically into 250 ml Erlenmeyer flask containing 45 ml of sterile inoculum medium, Glucose-2.0 g/l, Casein -0.5 g/l, Peptone-0.5 g/l, Yeast extract-0.5 g/l, Salt solution -50 ml.

Salt solution containing following the composition of salt, KH2PO4 -5.0g/l, MgSO4.7H2O- 5.0 g/l, FeSO4.7H2O - 0.1 g/l, pH-7.0.

The flask was kept in an incubator shaker at 220 rpm at 370C. The content of the flask was centrifuged at 3000rpm for 10mins and the supernatant was decanted. The cell pellet was washed thoroughly with sterile 20 g/l KCl2 solution, followed by NaCl2 solution and sterile distilled water subsequently. Finally the cell mass was suspended in sterile NaCl2 solution (9 g/l). This cell suspension was used as inoculum for fermentation as well as free cell fermentations.

Determination of Protease Activity

Azocoll method of Chavira et al.,(1984) and expressed in U/ mg.

Effect of pH Protease Activity

To measure the effect of pH on enzyme activity, Azocoll assays were performed using filtered supernatant and 10mM potassium phosphate buffer at pH 4.5, 8 and 9.

Effect of temperature and pH on Protease Production

The effect of temperature and pH on protease production was studied by cells were grown in fermentation media set at different temperatures 37, 45, 50, 55, 60 and 650C and different pH 5, 6, 7, 8, 9, and 10. Protease production was monitored at 6 hrs intervals over a 72 hrs fermentation period through assay of protease activity.

Whole Cell Immobilization

Whole cell immobilization of Terediobacter turnirae was done with different matrices such as calcium alginate, k- carrageenan, polyacrylamide, agar-agar and gelatin gel (Johnsen & Flink, 1986; Veelken & Pape, 1982). The reusability of immobilized cells protease production under repeated batch fermentation condition was also studied.

Protease Assay

Protease activity was assessed by the modified procedure based on the method of Tsuchida et al.,(1986)

Ammonium Sulfate Precipitation

To determined the precipitation fraction that contained the largest percent of protease activity. The protease activity and the protein concentration was measured and specific activity was calculated. The 20% fractions with the higher protease activity were determined.

III. RESULT AND DISCUSSION

a) Effect of Ph on Protease Activity

Azocoll was used as a protease substrate to assay the highest specific protease activity of bacteria Terediobacter turnirae. The pH 8 showed the highest protease activity (100%) compared to pH 9 (73 %). Whereas pH 4.5 showed very least protease activity (8%). These results indicated that Terediobacter turnirae protease was more active at an alkaline pH. Alkaline protease producetion has been reported in three Bacillus species, B. maceram, B. licheniformis and B. subtilis at an optimal pH of 8.0 (Olajuyigbe & Ajele, 2005).

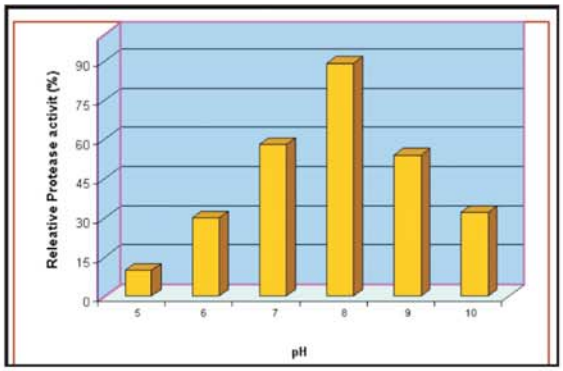

Fig. 1. Effect of Ph on Protease Production

b) Effect of Temperature and Ph on Protease Production

Physical parameters such as pH and temperature are used to study the protease production. The experiments were carried out at different pH (5, 6, 7, 8, 9, &10) and at different temperature range 20 0C to 70 0C. Maximum protease production was found at pH 8.0 and at temperature 600C. The results are shown in Fig. 1 & 2. The temperature range of activity of protease of Burkholderia, Pseudomonas and Bacillus species can range from as low as 40C to as high as 600C (Mitchell & Marshall, 1989; Stepaniak et al., 1982; and Olajuyigbe & Ajele, 2005).

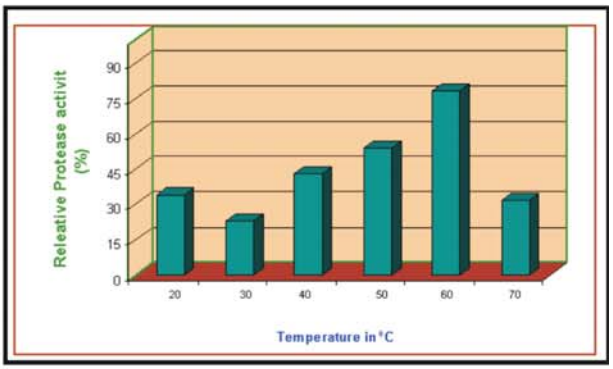

Fig. 2. Effect of Temperature on Proteas Production

C) Protease Production by immobilized cells in various matrices by Entrapment Technique

The protease production by immobilized cells in alginate matrix was found to be higher followed by k-Carrageenan (Fig.3). The production of alkaline protease improved significantly with increasing alginate concentration and reached a maximum enzyme yield of 8000 U/ml at 25-g/l-alginate concentration (Beshay, 2003). Adinarayana et al., (2005) reported that the calcium alginate is effective in protease production compared to the other matrices. Using k-Carrageenan, immobilized Brevibacterium flavum attained high stability against several denaturing chemicals (Ramkrishna & Prakasham, 1999).

Anna et al., (2003) reported that the use of agar entrapped cells of Bacillus circulans ATCC 21783 for cyclodextrin glucanotransferase production in fluidized bed reactor lod to enzyme activity (180 U/ml) after 24 hours of cultivation. In this present investigation the protease production in agar-agar was 376 U/ml.

Polyacrylamide was successfully used for immobilization of many enzyme systems (Kim et al., 1994). It was also used for the immobilization of cells for the production of other primary metabolites. The maximum protease production was 354 U/ml and 325 U/ml for polyacrylamide and gelatin respectively . Probably both glutaraldehyde (used for cross-linking with gelatin) and polyacrylamide monomers were toxic for the cells.

d) Repeated batch fermentation with immobilized cells in various matrices by Entrapment Technique

The amount of enzyme production with types of immobilized cells gradual decrease in protease production from the first batch onwards was observed (Fig.4). The beads were disintegrated during the 10th batch operation. Thus the repeated batch fermentation with calcium alginate beads was successfully run for 9 batches. Bandyopadhyay et al., (1993) studied erythromycin production by Streptomyces erythreus entrapped in calcium alginate beads and obtained efficient productivity of erythromycin. They could conduct repeated batch fermentation successfully (each batch 48 hours) for 12 batches (30 days). Similarly, Farid et al., (1994) reported that a good level of oxytetracycline was produced for a period of 28 days (7 batches) using Streptomyces rimosus cells immobilized in 4 % calcium alginate.

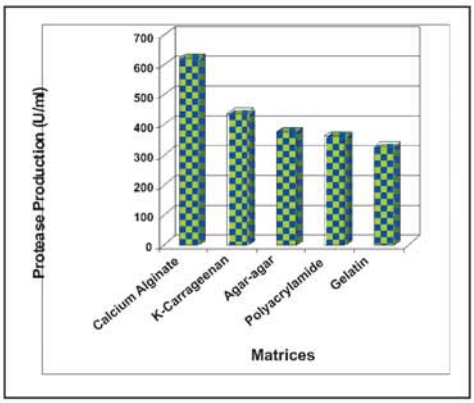

Fig. 3. Protease Production by iImmobilized cells in various Matrices by Entrapment Technique

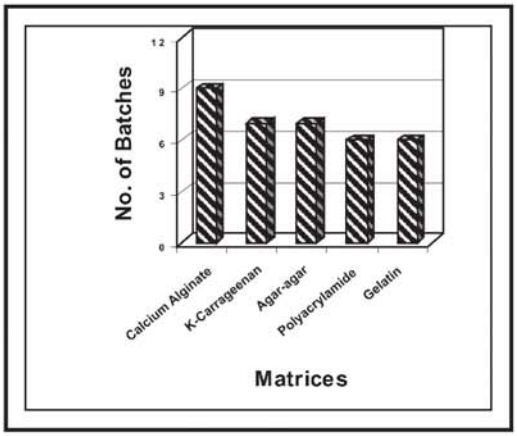

Fig.4. Repeated Batch Fermentation with immobilized cells in various matrices by Entrapment Technique

The immobilized cells entraped in k-carrageenan, agar-agar, polyacrylamide, and gelatin were also used for repeated batch fermentation. The behaviour of these systems were similar to alginate-immobilized cell. With all these systems, relatively low enzyme titers were observed when compared to alginate-immobilized cells and the gel cubes disintegrated after 6 batches of fermentation

E) Ammonium Sulfate Precipitation

Ammonium sulfate precipitation was performed as the first step of protease purification. Ammonium sulfate fractions collected at levels of saturation of 20%(w/v) were taken and assayed for specific protease activity after dialysis. Fraction collected at 40-60 % and 60-80% ammonium sulfate contained the highest percent of specific protease activity, with a total of 68.8% and accounted for 29 % at the total protein concentration (Table.1).

Ammonium sulfate purification increased the protease activity 20 fold compared to the activity present in the unconcentrated culture supernatant. This precipitation

step also decreased the overall protein concentration (70-90%) in the precipitation fraction containing the highest protease activity.

Table. 1. AMMONIUM SULFATE PRECIPITATION

Sl.No.	Salt added	Percent of enzyme specific activity	Percent of total protein concentration
1	0 - 20%	5.6 %	20 %
2	20 - 40%	18.3 %	15 %
3	40 - 60%	42.6 %	15 %
4	60 – 80 %	26.4 %	14 %
5	80 – 100%	5.1 %	17 %
6	100% +	8.7 %	25 %

IV. CONCLUSIONS

This study showed higher protease activity of Terediobacter turnirae at an alkaline pH. In the present investigation, it was found that optimum pH to be 8 and temperature to be 60 0C.

The alginate matrix was found to be better than other matrixes. In addition, the alginate matrix is less expensive, nontoxic, and preparation of biocatalyst involves mild conditions, which is an added advantage. Ammonium sulfate purification increased the protease activity 20 fold compared to the activity present in the unconcentrated culture supernatant.

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