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Research article

OPTIMIZATION OF MEDIA CONSTITUENTS FOR THE PRODUCTION OF ALKALINE PROTEASE FROM *BACILLUS LICHENIFORMIS*

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ABSTRACT: Production of alkaline protease by *Bacillus licheniformis* has been investigated under submerged fermentation. The physical and chemical parameters influencing submerged fermentation were optimized. The effect of incubation time, temperature, pH, carbon sources and nitrogen sources and additional nutrients on the production of alkaline protease was characterized. The optimum conditions for the protease production by *Bacillus licheniformis* were found to be at pH 9.0 and temperature at 40ºC. The outcome of carbon and inorganic nitrogen sources on protease production proved that glucose and casein were the effective medium ingredients for *Bacillus licheniformis* respectively. The maximum amount of protease production was recorded in medium supplemented with ammonium sulphate. Among the tested metal ions, the level of protease yield was found to be high in medium supplemented with magnesium chloride. The protease production was amplified in the presence of 1.5% sodium chloride. The extreme stability towards Triton X-100, Tween 20 and SDS was observed in *Bacillus licheniformis* alkaline protease.

Key words: Protease, Bacillus *licheniformis* and surfactant.

INTRODUCTION

Proteases comprise a class of industrial enzymes, which alone form approximately 60% of the total world-wide enzyme production (Chu, 2007). Among the various types of proteases, the bacterial proteases play an important role in biotechnological processes and other industrial applications especially in medical field. The production of alkaline protease has special interest due to the manufacture of detergents, food, clinical and leather industry (Saeki *et al*., 2007, Sankaralingam *et al*., 2012). Now a day's number of studies has been conducted to characterize alkaline protease from different bacterial strains, fungal strains and other microbial sources. Even though, alkaline proteases have been paying attention due to low stability towards surfactants, metal ions and production cost (Joo and Chang, 2005).

Protease yields are influenced by some physical factors such as aeration, inoculum size, pH, temperature and incubation time (Hameed *et al*., 1999) and biological factors such as the genetic nature of the organism, which are influencing the metabolic and biochemical behavior of the microbial strain (Kumar and Takagi, 1999; Elliah *et al*., 2002). Until now, there is no distinct medium has been established for the maximum production of any secondary metabolite and any other substances, because the genetic diversity exist in microbial sources causes each organisms or strain to have its own special conditions for the maximum protease production. Therefore, it is worth to have a detailed investigation on newly isolated microbial strain for production pattern under different environmental conditions and in an optimized pattern to achieve maximum production (Elliah *et al*., 2002). Though the protease production by bacterial strains was documented well, the protease productions by agricultural bacterial isolates are still required. Hence the present work was undertaken to investigate the protease production by the agricultural bacterial isolate *Bacillus licheniformis*.

MATERIALS AND METHODS

Isolation and screening of proteolytic bacterial strain

Protease producing bacterial strain was isolated from Alagar hills, Tamilnadu, India. Then the isolated strain was identified by standard procedures described in Bergy's manual of determinative bacteriology. The identified organism was tested for protease production on skim milk agar plates. After 24 h of incubation at 35^{o}C, the proteolytic activity was confirmed by clear zone formation around the bacterial growth. The protease activity in the liquid medium was assessed first by enriching the bacteria in enrichment medium containing beef extract (0.3 %), peptone (0.5 %), NaCl (0.5 %) and glucose (0.5 %) at pH 7 for 24 h and then 10 % of this enriched culture was inoculated in 250 ml flask containing 45 ml Basal medium containing (g/l) - $(NH_4)_2SO_4$ – 2g ; K_2HPO_4 – 1g ; KH_2PO_4 – 1g ; $MgSO_4.7H_2O$ – 0.4g ; $MnSO_4.H_2O$ – 0.01g ; $FeSO_4.7H_2O$ – 0.01g ; Yeast extract – 1g ; Peptone – 10g at pH 7. The culture was then incubated for 2 days by shaking at 35^{o}C. The cells were then harvested by centrifugation at 10000 rpm for 15 min and the supernatant was used for further protease assay.

Protease assay

The assay system consists of following ingredients such as 1.25 ml Tris buffer (pH 7.2), 0.5 ml of 1% aqueous casein solution and 0.25 ml culture supernatant. Appropriate controls were also made with out enzyme source. The mixture was incubated for 30 min at 35^{0}C. Then 3 ml of 5% TCA was added to this mixture and placed at 4^{0}C for 10 min to form precipitate. Then it was centrifuged at 5000 rpm for 15 min. From this, 0.5 ml of supernatant was taken, to this 2.5 ml of 0.5M sodium carbonate was added, mixed well and incubated for 20 min. Then it was added with 0.5 ml of folin phenol reagent and the absorbance was read at 660 nm using UV-Vis Spectrophotometer (TECOMP 8500). The amount of protease produced was estimated and expressed in microgram of tyrosine released under standard assay conditions.

Optimization of media constituents

1. Effect of carbon sources on protease production

The effect of different carbon sources on protease production was studied by using various carbon sources such as dextrin, arabinose, sucrose, fructose, glucose, raffinose, starch, sorbitol and lactose were supplemented individually at 0.5% concentration in the optimized basal medium inoculated with 2% of 24h broth culture.

2. Effect of inorganic and organic nitrogen sources on protease production

The effect of nitrogen sources was studied by using various types of nitrogen sources such as soy meal, beef extract, peptone, urea, yeast extract, skim milk powder, casein sodium nitrate, potassium nitrate, ammonium chloride, ammonium sulphate and ammonium nitrate. They were tested individually at 0.5% concentration in the optimized carbon sources added to basal medium inoculated with 2% of 24h culture broth.

3. Effect of sodium chloride on protease production

The effect of NaCl on protease production was calculated by the addition NaCl to the production media. The experiment was carried out individually at 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5% NaCl in optimized basal medium inoculated with 2% of 24h culture broth.

4. Effect of surfactants on protease production

The effect of surfactant on protease production was calculated by using 5 different surfactants such as Tween 20, Tween 80, Triton X 100, poly ethylene glycol (PEG) and sodium dodecyl sulphate (SDS). The selected surfactants were incorporated individually into the optimized medium at 0.2% concentration and the medium without surfactant was focused as control.

5. Effect of metal ions on protease production

To assess the effect of metal ions on protease production by using various kinds of metal ions such barium chloride, copper sulphate, zinc sulphate, magnous chloride, ferrous sulphate, calcium chloride, mercuric chloride, ferric chloride and EDTA were used.

6. Effect of pH and Temperature on protease production

The effect of pH on protease production by the experimental microorganism was determined by using different pH buffers in the assay medium. The assay was carried out individually at various pH such as 3, 4, 5, 6, 7, 8 and 9. Simultaneously, the effect of temperature on protease production was studied by incubating the enzyme and substrate solution at various temperatures such as 10, 20, 30, 40, 50, 60, 70 and 80^{0}C.

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RESULTS

Isolation and screening of protease producing bacterial strains

Four different groups of colonies with varying morphology were detected in soil samples. Among the detected four bacterial colonies only one was the dominant protease producer and the zone formation around the bacterial growth showed.

Identification of protease positive colony

Depending upon on the morphological, cultural and biochemical characteristics the suspected colony was identified as *Bacillus licheniformis* by the following standard keys of Bergey's Manual of Determinative Bacteriology (Table-1) and the isolated bacterial strain was screened for protease producing ability on skim milk agar. Hence the strain was identified as a protease producer and it was taken for further experimental analysis (Table 1).

Table-1: Biochemical characteristics of the *Bacillus licheniformis*

Biochemical tests	**Results**
Gram's staining	Gram positive *Bacillus licheniformis*
Endospore staining	Sub-terminal spores
Motility	+
Carbohydrate fermentation test	
a. D-glucose	+
b. Mannitol	+
c. Lactose	-
d. Sucrose	+
Indole production	-
Methyl red test	-
Voges-Proskauer test	+
Citrate utilization test	+
Starch hydrolysis	+
Gelatin hydrolysis	+
Casein hydrolysis	+
Urease test	-
Catalase test	+
Oxidase	-
Nitrate utilization test	+

+: Positive Results **- : Negative Results**

Effect of incubation time

Figure 1 shows incubation interval on the protease production indicated that the 24 hours of incubation was suitable for *Bacillus licheniformis*. A gradual reduction of protease yield with increased in incubation time.

Effect of carbon sources

The effect of carbon sources on protease production by *Bacillus licheniformis* after 24 h of incubation period indicated that, it was maximum in glucose supplemented medium (Fig. 2).

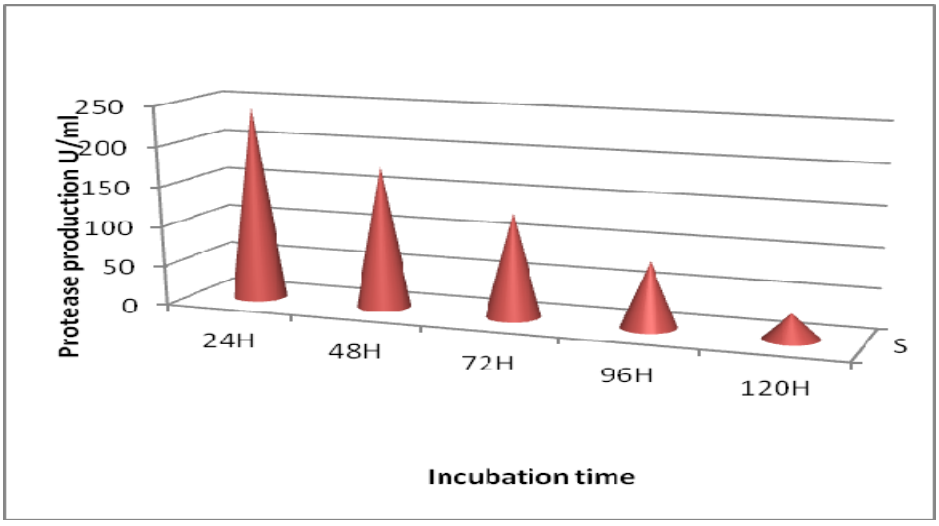

Fig. 1: Production profile of protease produced by *B. licheniformis* in various incubation time

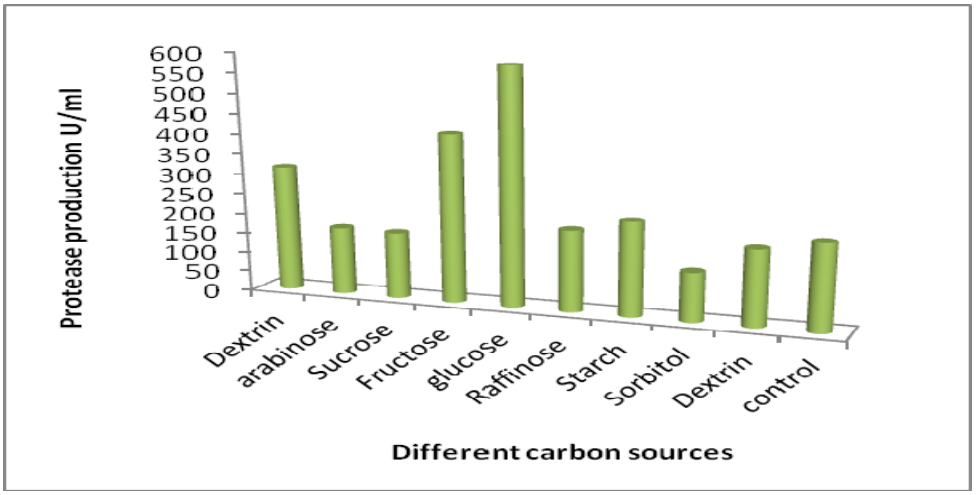

Fig. 2: Result of various carbon sources on protease production produced by B. *licheniformis*

Effect of organic nitrogen sources

Among the tested organic nitrogen sources, the maximum amount of protease production was registered in casein added medium after 24 h of incubation (Fig. 3).

Effect of inorganic nitrogen sources

Among the tested inorganic nitrogen sources, the maximum amount of protease production was registered in ammonium sulphate added medium after 24 h of incubation (Fig. 4).

Effect of various concentration of NaCl

The effect of NaCl on protease production by *Bacillus licheniformis* inferred that, it was high in 1.5% concentration added medium. The lowest amount of enzyme production was recorded in 3.5% NaCl added medium (Fig. 5).

Effect of surfactant

The result of surfactant on protease yield inferred that the highest yield was recorded in PEG added medium (Fig. 6).

Effect of metal ions

Among the tested metal ions, the maximum amount of enzyme production was recorded in magnesium chloride added medium after 48 h of fermentation. On the other hand, the minimum amount of enzyme production was recorded in mercuric supplemented medium (Fig. 7).

Effect of pH and temperature

The effect of pH on protease production revealed that it was maximum at pH 7.0 and minimum at pH 9 (Fig. 8). Similarly in the experimentation on the effect of temperature (Fig. 9), the maximum protease production was recorded in 40^0C and minimum was registered at 80^0C.

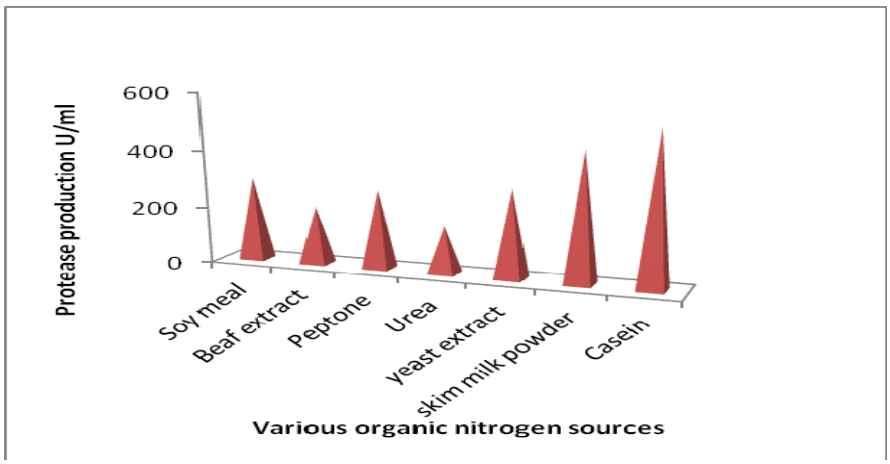

Fig. 3: Result of various organic nitrogen sources on protease production by *B. licheniformis*

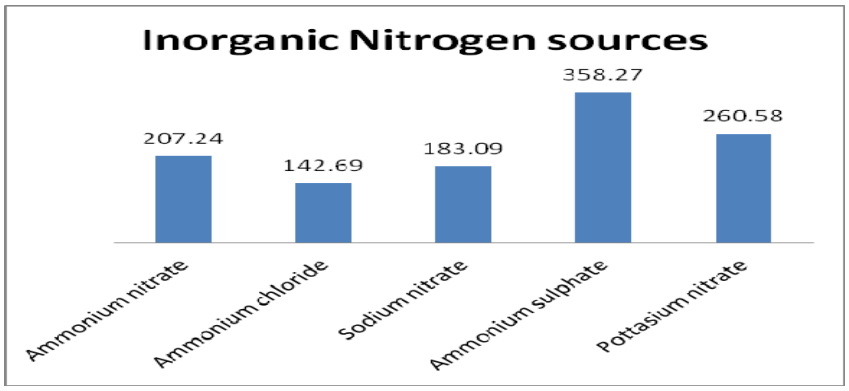

Fig. 4: Result of various inorganic nitrogen sources on protease production by *B. licheniformis*

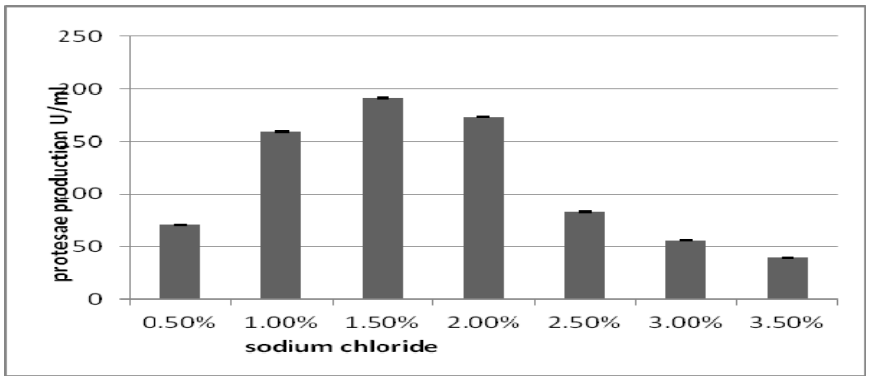

Fig. 5: Production profile of protease in medium supplemented with various concentrations of sodium chloride

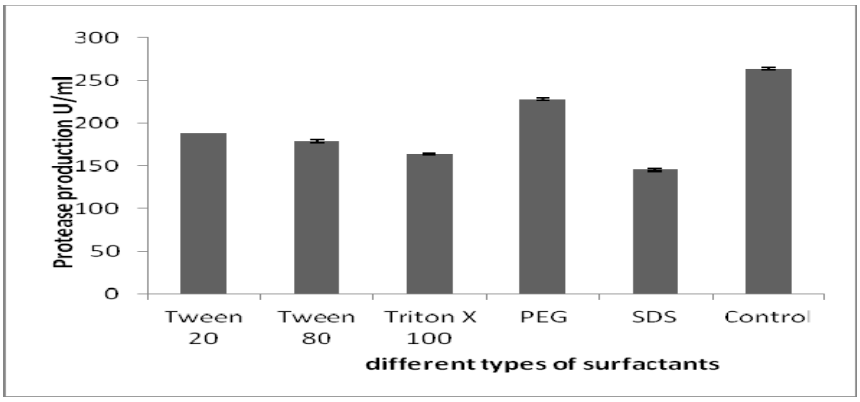

Fig. 6: Result profile of protease in medium supplemented with various kinds of surfactants

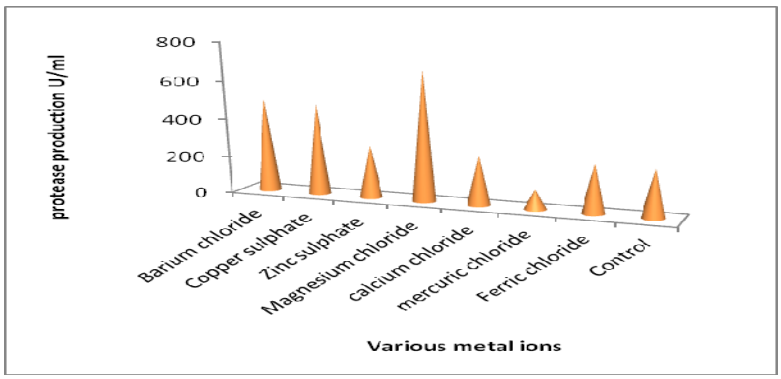

Fig. 7: Production profile of protease in medium supplemented with various kinds of metal ions

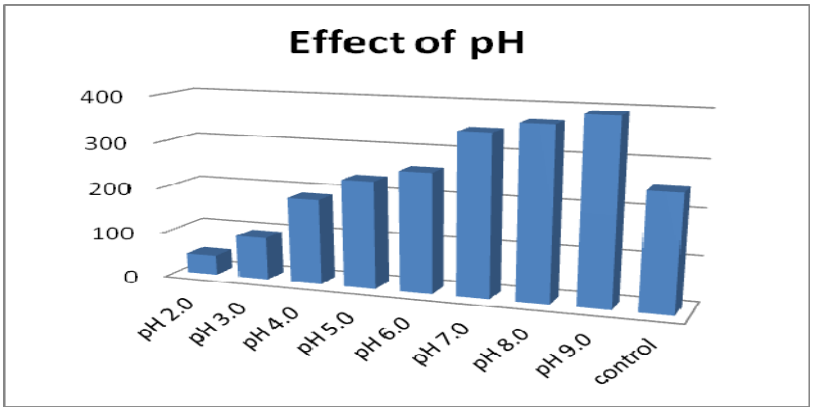

Fig. 8: Effect of various pH on protease production.

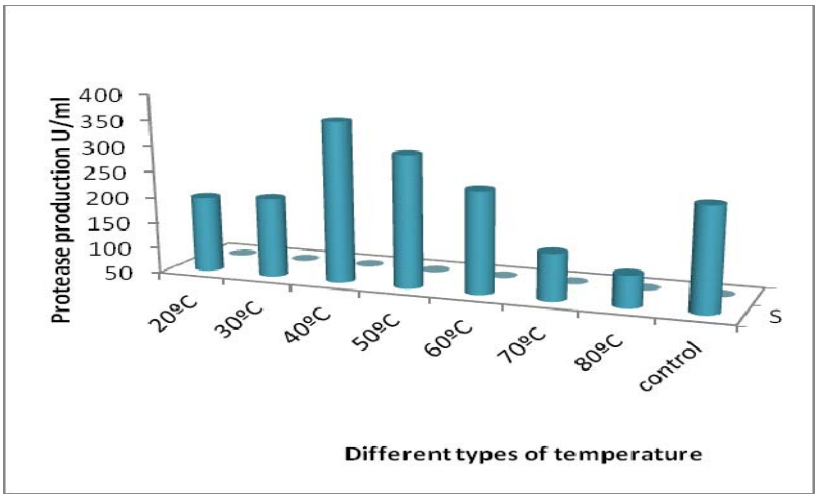

Fig. 9: The effect of various temperatures on protease production.

DISCUSSION

Protease is the essential ingredients of all forms of life on earth including prokaryotes, fungi, plants and animals. Among the various factors influencing the protease production, the nutrient sources such as carbon and nitrogen have thoughtful effect on microbial proteases yield. The present study revealed that maximum amount of protease production was recorded in casein added medium. This is because casein is not only served as nitrogen source but also has energy resources such as carbohydrates, free amino acids. The results on the casein improved protease production by *Bacillus licheniformis* also supports the previous studies on casein aided higher protease production by *Virgibacillus pantothenticus* (Gupta *et al*., 2007), *Bacillus flexus* (Sankaralingam *et al*., 2014). The effect of various carbon sources on protease production indicated that glucose gave higher protease production when compared to other carbon sources supplied. In consistence with this present study, bacteria such as *Shigella* sp. (Sankaralingam *et al*., 2011) and *Bacillus clausii* (Joo *et al*., 2003) were also reported higher protease production in glucose supplied medium. The effect of various surface active agents on the fermentative production of protease revealed that Triton X 100 was found to increase the protease production. This is correlated with the report of Joo and Chang (2005) that the protease from *Bacillus clausii* and *Bacillus* sp. retain their activity with different surfactants such as Triton X 100, Tween 20 and SDS. Results on the effect of metal ions and trace elements on protease production revealed that the mercury, zinc, copper and calcium ions had profound effect on the protease production by *Bacillus licheniformis*. Sumantha *et al*. (2005) and Abd Rehman, (2005) reported that, some bacteria and fungi showed maximum enzyme production with metal ions such as Ca^{2+} and Mg^{2+} .

pH in the fermentation media is the main factor deciding the effective fermentation process. The results indicated that the protease produced by *Bacillus licheniformis* was maximum at pH 7.0. Mahendiran *et al*. (2010) reported that the maximum alkaline protease production was recorded in pH 10 by *Bacillus aquimaris*. The effect of various incubation temperatures on protease production by the tested candidate species inferred that 40^0C was found to be a suitable temperature range for this bacterium.

This was supported by the studies of Gupta et al. (2007) and Samarntarn *et al*. (1999), where the protease from *Virgibacillus pantothenticus* has its maximum activity with the temperature range between "30 – 50^0C". The effect of sodium chloride showed that this bacterium could use the salt range between 0.5% to 1.5% for maximizing the protease production and it was remarkably high in 1.5% sodium chloride supplied medium. Mahendran *et al*. (2010) reported that the protease production by marine bacterium *Bacillus aquimaris* absolutely require 1.5% NaCl supplemented medium.

In the present study, the maximum amount of protease production was observed in 24h incubation interval. The organisms maintained a slow growth up to 10h, after which the growth was exponential up to 40 h followed by stationary phase. The production of protease nearly corresponded maximum in early stationary phase (Patel *et al.*, 2005).

CONCLUSION

The aim of the work was to evaluate the protease producing ability of *Bacillus licheniformis*. The isolated strain was identified by the biochemical characteristics. Results indicated that the optimum condition for the protease production by the candidate bacterium was found to be at pH 9.0 and temperature at 40ºC. Supplementations of maltose and casein acid hydrolysate were the suitable substrates for protease production. Among the tested metal ions and surfactants, calcium chloride and Triton X 100 gave the better enzyme production.

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