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Research Paper

Production, Optimization, Partial Purification and Immobilization of protease from Newly Isolated Bacterial Strain T_3

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ABSTRACT

The research was conducted for protease production and its usage after immobilization. Enzyme production was optimized using submerged fermentation technique for different ranges of pH and different concentrations of different carbon and nitrogen sources. Fermentation media was incubated for 48 h at 37°C temperature with agitation speed of 200 rpm. Protease production (6.4 U/ml/min) from bacterial strain T_3 was optimized at 2.0% soybean meal, 1.5% glucose and 8 pH. Protease was partially purified with 70% ammonium sulphate and immobilized on different supports by physical adsorption and their activities checked. When partially purified protease was immobilized on Amberlite (XAD 761) then increase of protease production was 208.33% (25 U/ml/g support) for strain T_3. When partially purified protease was immobilized on Duolite (A568), the increase of protease production was 225% (27 U/ml/g support) for strain T_3 and when partially purified protease was immobilized on Lewatit (VPOC, 1600), the increase of protease production was 541.66% (65 U/ml/g support) for strain T_3. When partially purified protease was immobilized on Pentynyl Dextran (NT4L360), the increase of protease production was 3483.33% (418 U/ml/g support) for strain T_3. This immobilized protease with 35-fold increase in its activity may be used in food industry, pharmaceutical industry, bioremediation, detergent industry and textile industry etc.

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Key words: Protease production, partially purified and immobilized protease, isolated bacterial strain T_3.

INTRODUCTION

Proteases, also called proteinases or peptidases, working as molecular knives, break long amino acid sequences necessary for the synthesis of proteins, regulating their size, composition, shape, turn-over and ultimate destruction into fragments (Seife, 1997). Submerged fermentation technique is most widely used for protease synthesis and 90% of proteases are synthesized through this technique (Gupta, 2002; Gonz, 2003; Olker, 2004). There are some specific conditions for maximum synthesis of proteases. A balance has to be maintained for optimization of the media composition. Hence, cost effectiveness of the media is an essential factor for the development of the production media (Biesebeke, 2002).

There are some optimizing factors for maximum enzyme synthesis like media component specifically carbon and nitrogen sources, temperature, pH, incubation time, agitation and inoculums density (Chellappan et al., 2006; Chi et al., 2007). Organic and inorganic forms of nitrogen are metabolized for the synthesis of amino acids, nucleic acids, proteins and components of the cell wall (Kole et al.,

1988). Different strategies at industrial level are used by many biochemical and process engineers for the synthesis of protease from micro-organism (Hameed et al., 1999).
Enzymes immobilization simply means the enzyme is bound to restrict its mobility in a fixed space (Shuler and Kargi, 2002). Some useful techniques like covalent binding,ionic binding, adsorption, entrapment, cross-linking and encapsulation are used for immobilization of proteases (Church et al., 1992). In physical adsorption, enzymes and matrixes are linked together through hydrogen bonding, van der Waals forces, or hydrophobic interactions. In ionic bonding, enzymes and matrixes are linked with salt linkages. Enzymes catalytic activity is preserved by this method of physical adsorption. Enzyme leakage takes place when enzyme-matrix interaction becomes relatively weak (Bahulekar et al., 1991). Stability of pH may change after enzyme immobilization (Goldstein, 1972; Goldman et al., 1968). Immobilization of enzymes has the ability to change its catalytic activity or thermal stability (Trevan, 1980).

Intrinsic activity of immobilized enzyme changed after immobilization. Operational stability of enzyme increased due to immobilization of the enzyme (Blanco et al., 1989). Immobilized enzyme has the ability to recover its catalytic activity (Jakubke, 1994).

MATERIALS AND METHODS

Inoculum preparation

Nutrient broth (0.8 g) was weighed and dissolved it in 50 ml of distilled water. Water was continuously added in the solution until it was marked with 100 ml of that solution. 0.8% of 100 ml flask of nutrient broth was autoclaved for 15 min at 15 lb/inch square pressure and 121°C temperature. Loopful of bacteria was then transferred to inoculum flask and kept in shaking incubator with 200 revolutions per minute agitation speed at 37 centigrade temperature for 24 h.

Fermentation medium

Submerged fermentation technique was used for protease synthesis. 2 g of soybean meal, 1.5 g of glucose, 2 g of peptone, 0.1 g $(NH_4)_2SO_4$, 0.1 g KH_2PO_4 and 0.5 g sodium carbonate were weighed and then dissolved in 50 ml of distilled water. Water was continuously added in the solution until it was marked with 100 ml of that solution. The cotton plugged flasks was then subjected to sterilization in an autoclave for 15 min under 15 lb/inch square pressure and 121°C temperature. 0.5 ml of inoculum was added in the fermentation media and kept in a shaking incubator with 200 revolutions per minute speed at 37°C temperature for 48 h. The centrifugation of the test tube sample with 6000 revolutions per minute was carried out for 10 min.

Assay of protease

The activity of protease was checked through a special procedure introduced by McDonald and Chen (1965). 1.0 ml protease extract (which was obtained after centrifugation at 6000 revolutions per minutes for 10 min) was poured in the test tube and 4.0 ml of 1.0% casein added in same test tube. The test tube was then placed in an incubator for one hour at 37°C temperature. After that, 5 ml of 5% TCA was added in the test tube. After 30 min, the test tube sample was passed under the process of centrifugation (6000 revolutions per minute) for ten minutes. One milliliter of supernatant was mixed with alkaline reagent (5 ml). Then, 1 ml of 1 N sodium hydroxide was mixed in the test tube. The test tube sample was allowed to react with each other for 10 min. Folin and Ciocalteau reagent (0.5 ml) was mixed; as a result, blue color was produced. Blue colour appeared after 30 min in the test tubes. The optical density of the mixture was read at 700 nm "UV/VIS Spectrophotometer (Cecil-CE7200-Series, Aquarius, UK)". Activity of enzyme was expressed as: One unit of protease defined as the amount of enzyme required to produce an increase of 0.1 in optical density under optimal defined conditions.

Preparation of 1% casein solution

Casein (1 g) was weighed and then dissolved in 50 ml of distilled water. Water was continuously added in the solution until it a mark of 100 ml of that solution was reached. The beaker with casein solution was placed on hot plate. NaOH (1 N) was added (approximately, 1 ml) in casein solution with continuous stirring.

Preparation of 5% TCA solution

TCA (5 g) was weighed and then dissolved in 50 ml of distilled water. Water was continuously added in the solution until it was marked with 100 ml of that solution.

Preparation of 1N NaOH solution

NaOH (4 g) was weighed and then dissolved it in 50 ml of distilled water. Water was continuously added in the solution until it attained a mark of 100 ml of that solution.

Preparation of alkaline reagent

Sodium potassium tartarate (1 ml of 2.7%) and Copper sulphate (1 ml of 1%) was dissolved in 50 ml of 2%

sodium carbonate solution and water continuously added in that solution until it was marked with 100 ml of that solution.

Preparation of 2.7% sodium potassium tartarate solution

Sodium potassium tartarate (2.7 g) was weighed and then dissolved in 50 ml of distilled water. Water was continuously added in the solution until it was marked with 100 ml of that solution.

Preparation of 2% of sodium carbonate solution

Sodium carbonate (2 g) was weighed and then dissolved in 50 ml of distilled water. Water was continuously added in the solution until a mark of 100 ml of that solution was attained.

Preparation of 1% Copper sulphate solution

Copper sulphate (1 g) was weighed and then dissolved in 50 ml of distilled water. Water was continuously added in the solution until a mark of 100 ml of that solution was attained.

Optimization of enzyme production

Different parameters were optimized for the synthesis of bacterial protease.

Effect of different sources of carbon on protease production

To check the effect of different sources of carbon on protease synthesis, various carbon sources like glucose, starch, sucrose, fructose, lactose and maltose were added separately in the fermentation media. Same procedure was repeated for preparation and incubation of fermentation media under same condition as earlier described. Then, activity of protease was checked and compared with each other.

Effect of different carbon concentration on protease production

To check the effect of different carbon concentration on protease synthesis, various carbon concentrations like 0.5, 1, 1.5, 2, 2.5 and 3% was separately adjusted in the fermentation media respectively. Thereafter, same procedure was repeated for preparation and incubation of the fermentation media under same condition as earlier described. The activity of protease was checked and compared with each other.

Effect of different sources of nitrogen on protease production

To check the effect of different sources of nitrogen on protease synthesis, various nitrogen sources like wheat flour, mustard meal, cotton meal, almond meal and soybean meal was added in fermentation media separately.Then same procedure was repeated for preparation and incubation of fermentation media under same condition as described earlier. Then activity of protease was checked and compared with each other.

Effect of different nitrogen concentration on protease production

To check the effect of different nitrogen concentration on protease synthesis, various carbon concentrations like 0.5%, 1%, 1.5%, 2%, 2.5% and 3% was adjusted in fermentation media separately. Then same procedure was repeated for preparation and incubation of fermentation media under same condition as described earlier. Then the activity of the protease was checked and compared with each other.

Effect of pH on protease production

To check the effect of different pH values on protease synthesis, various pH values from 5.5 to 9.0 was separately adjusted in the fermentation media. The same procedure was repeated for preparation and incubation of the fermentation media under same condition as earlier described. The activity of the protease was later checked and comparisons with each other carried out.

Purification and concentration of protease

Purification and concentration was done by salting out the enzyme by addition of ammonium sulphate. $(NH_4)_2SO_4$ was continuously mixed with the enzyme at temperature of 4°C. The addition of $(NH_4)_2SO_4$ was continuous up to 70% of saturation and the solution kept in cool laboratory at 4°C temperature for ten minutes. After 30 min, the test tube sample was passed under the process of centrifugation (10000 revolutions per minute) for ten minutes. The precipitated enzyme (pellet) was redissolved in a minimum amount of 0.1 M Tris. HCl buffer solution and dialyzed. For the purpose of dialysis, 10 cm long and 25 mm wide dialysis tube was utilized. 10 ml of the dissolved pellets were then poured into the given dialysis

Figure 7: Immobilization of protease produced from bacterial strain T_3 on Amberlite (XAD 761).

tube and kept in 1000 ml of 0.1M Tris. HCl buffer solution with continuous strirring for 24 h at 4°C. During this period, buffer was refreshed 3 to 4 times. The purified and concentrated enzyme was used for immobilization studies.

Preparation of 0.1M Tris. HCl buffer

To prepare 0.1 M Tris-HCl buffer solution, 12.11 g of Tris (hydroxymethyl)-aminomethane (commonly called as Tris-base $C_4H_{11}NO_3$) was weighed then dissolved in 500 ml of distilled water. Water was continuously added in the solution until it a mark of 1000 ml of that solution was attained. After that hydrochloric acid was added drop by drop to adjust its final pH 8 with 0.1 N hydrochloric acid and 0.1 sodium hydroxide (Robinson, 1988).

Preparation of 0.1 N HCl

HCl (8.3 ml of 37%) was dissolved in 50 ml of distilled water. Water was continuously added in the solution until a mark of 100 ml of that solution was attained.

Preparation of 0.1 N NaOH

NaOH (0.4 g) was weighed and then dissolved in 50 ml of distilled water. Water was continuously added in that solution until a mark of 100 ml of that solution was attained.

Immobilizing supports

Different supports for immobilization (Figure 7 to 10) utilized are:

- Lewatit VPOC 1600 (Lanxess- Germany) (TuBraunschweig, Germany);
- Duolite A568 (Rohm amd Hass- France) (TuBraunschweig, Germany);
- Amberlite XAD 761(Rohm amd Hass- France) (TuBraunschweig, Germany);
- Pentynyl Dextran (NT4L360) Insitute Fur Lebensmittle Chemie (TuBraunschweig, Germany).

Physical adsorption (Immobilization technique)

Enzyme was immobilized on different supports thus; 0.5 g of each support was stirred with 5 ml of partially purified protease and kept in shaking water bath at the speed of 100 rpm at 37°C for 60 min and thereafter, centrifuged at 6000 revolutions per minute for 10 min. The supernatant was then used for protease activity (Minovska et al., 2005).

RESULTS AND DISCUSSION

Effect of different sources of nitrogen for production (U/ml) of protease from bacterial strain T_3

Different sources of nitrogen were optimized for the

Figure 8: Immobilization of protease produced from bacterial strainT_3 on Pentynyl Dextran (NT4L360).

Figure 9: Immobilization of protease produced from bacterial strainT_3 on Lewatit (VPOC 1600).

synthesis of protease from strain T_3. 1% of different nitrogen sources like wheat flour, mustard meal, almond meal, cotton meal and soybean meal were separately added in fermentation media (Figure 1). The same procedure was repeated for preparation and incubation of fermentation media under same condition as earlier described. The activity and production of protease of different nitrogen sources was then checked and compared with each other. Wheat flour, mustard meal, almond meal, cotton meal and soybean meal were shown to be 4.1, 4.2, 3.8, 4.0 and 4.8 U/ml of protease productions respectively. From the result, soybean meal (4.8 U/ml) was

Figure 10: Immobilization of protease produced from bacterial strain T_3 on Duolite (A568).

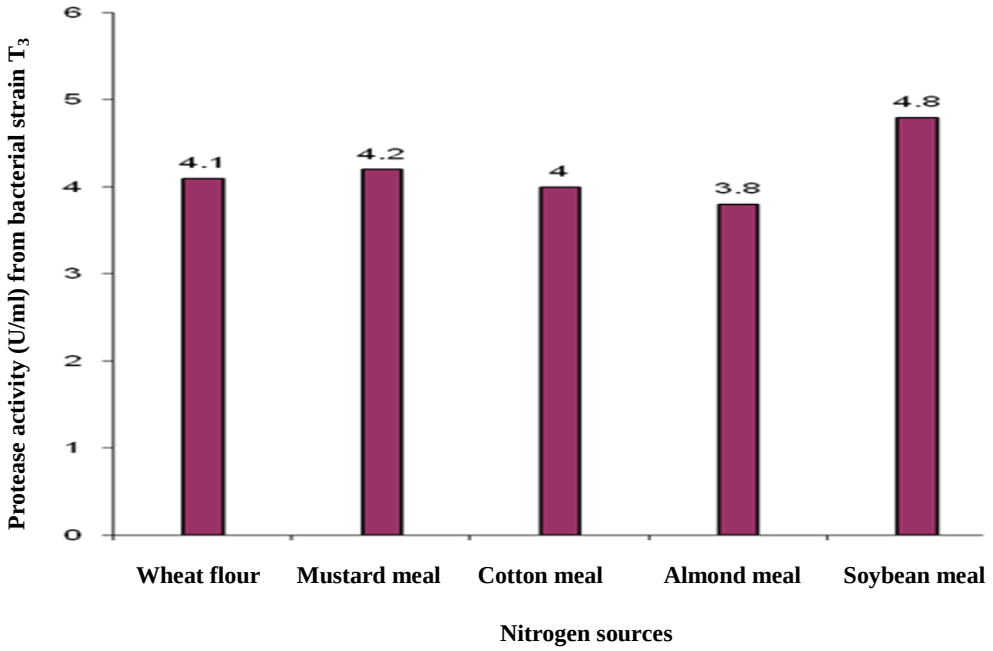

Figure 1: Effect of different sources of nitrogen for production (U/ml) of protease from bacterial strain T_3.

declared the best nitrogen source for bacterial strain T_3.

Effect of different soybean meal concentrations for production (U/ml) of protease from bacterial strain T_3

Nitrogen concentrations were optimized for the synthesis of protease from strain T_3. Different soybean concentrations of 0.5, 1, 1.5, 2, 2.5 and 3% were separately adjusted in the fermentation media respectively (Figure 2). The same procedure was repeated for preparation and incubation of fermentation media under the same condition as earlier described. The activity of protease was later checked and compared with each other. The protease

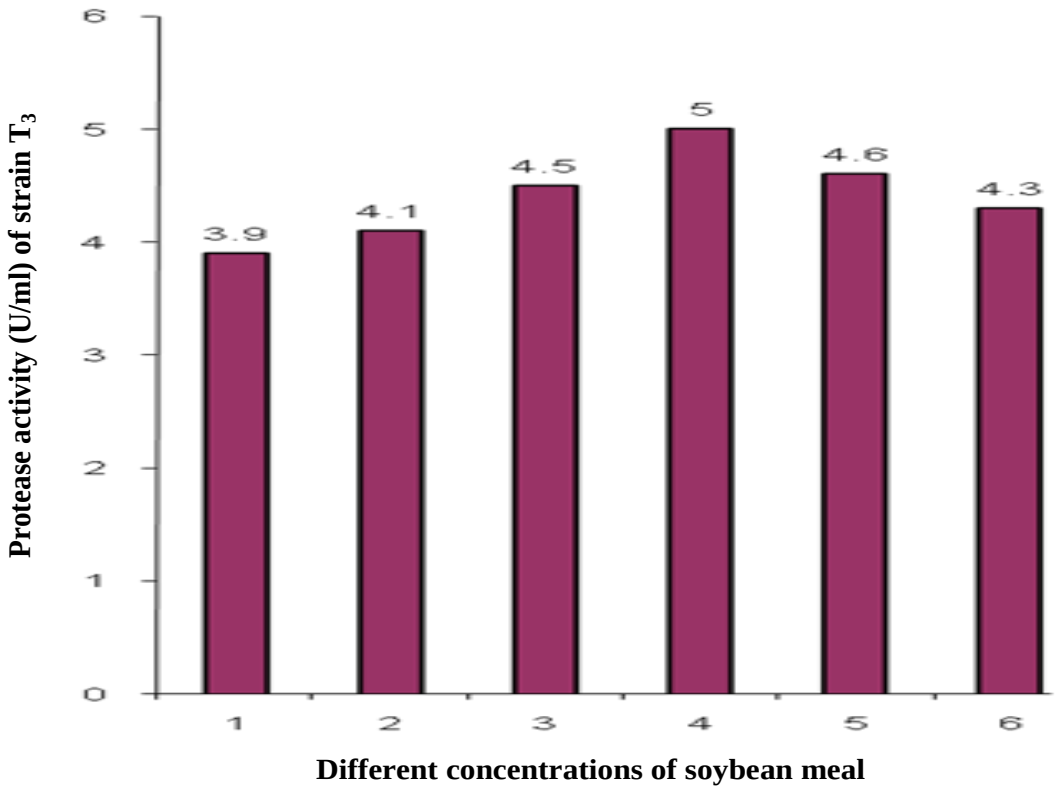

Figure 2: Effect of different soybean meal concentrations for production (U/ml) of protease from bacterial strain T_3. 1=0.5%, 2=1.0%, 3=1.5%, 4=2.0%, 5=2.5% and 6=3.0%. Where 1, 2, 3, 4, 5 and 6 represent the different concentrations of soybean meal.

production (U/ml) of different soybean concentrations was compared. As a result, 0.5, 1, 1.5, 2, 2.5 and 3% concentration of soybean meal showed 3.9, 4.1, 4.5, 5.0 4.6 and 4.3 U/ml of protease production respectively. From the analysis, 2% concentration of soybean meal (5.0 U/ml) was declared the best soybean meal concentration for bacterial strain T_3.

Effect of different sources of carbon for production (U/ml) of protease from bacterial strain T_3

Different sources of carbon were optimized for the synthesis of protease from strain T_3. 1% of different carbon sources like glucose, starch, sucrose, fructose, lactose and maltose were separately added in the fermentation media. The same procedure was repeated for preparation and incubation of fermentation media under the same condition as earlier described and the activity of protease checked and compared with each other. Thereafter, the protease production (U/ml) of different carbon sources was compared (Figure 3). Accordingly, glucose, starch, sucrose, fructose, lactose and maltose showed 5.4, 5.0, 4.7, 5.1, 4.9 and 4.8 U/ml of protease production respectively. This therefore resulted in glucose (5.4 U/ml) being the best carbon source for bacterial strain T_3.

Effect of different concentrations of glucose for production (U/ml) of protease from bacterial strain T_3

Different concentrations of carbon were optimized for the synthesis of protease from strain T_3. Different glucose concentrations like 0.5, 1, 1.5, 2, 2.5 and 3% were separately adjusted in the fermentation media (Figure 4) respectively. The same procedure was repeated for preparation and incubation of the fermentation media under the same condition as earlier described. Thereafter, the activity of protease was checked and compared with each other and the protease production (U/ml) of different glucose concentrations also compared. Consequently, 0.5, 1%, 1.5, 2, 2.5 and 3% concentration of glucose showed 5.0, 5.3, 6.0, 5.4, 5.0 and 4.9 U/ml protease productions respectively. This therefore resulted in 1.5 % concentration of glucose (6.0 U/ml) being declared the best glucose concentration for bacterial strain T_3.

Effect of different pH concentrations for production (U/ml) of protease from bacterial strain T_3

Different pH concentrations were optimized for the synthesis of protease from strain T_3. Different pH concentrations like 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9% were separately adjusted in the fermentation media

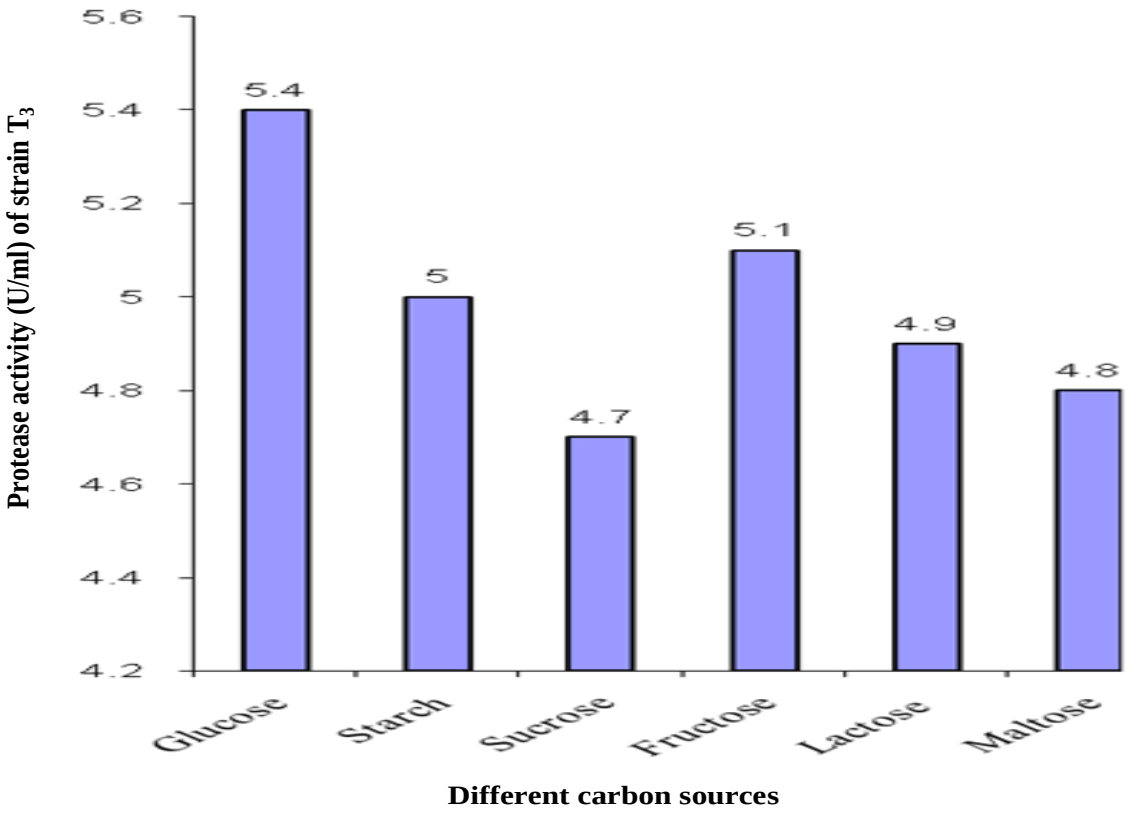

Figure 3: Effect of different sources of carbon for production (U/ml) of protease from bacterial strain T_3.

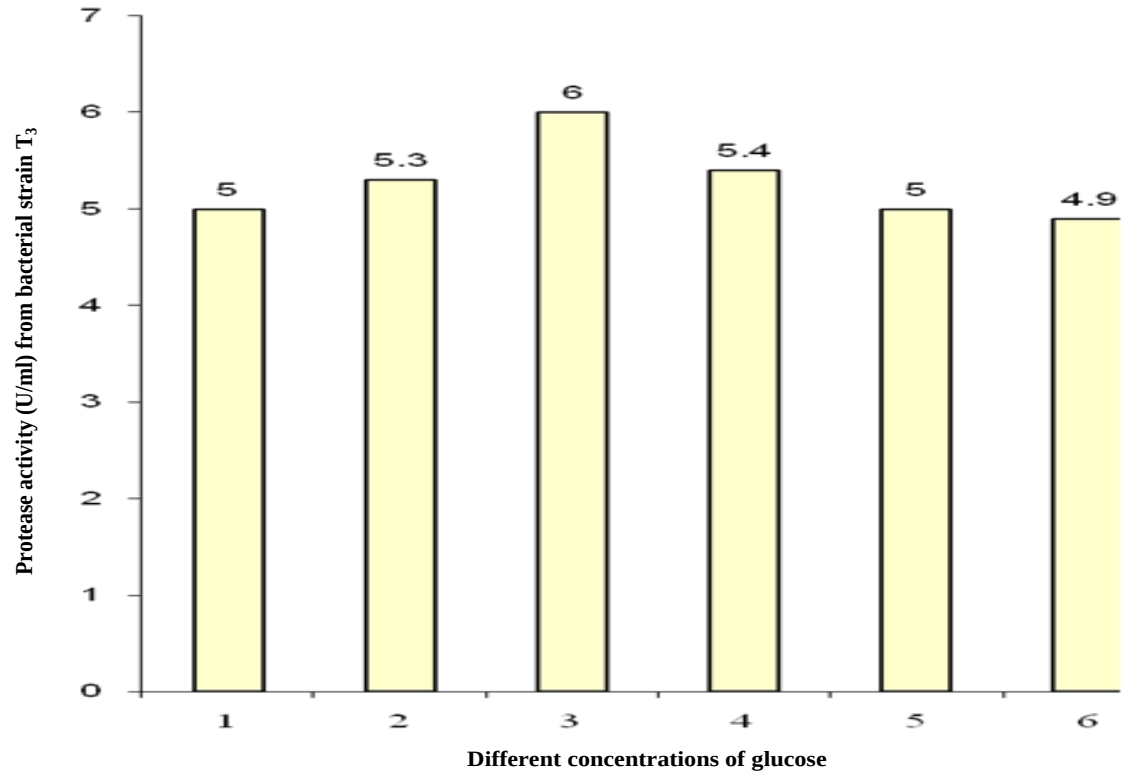

Figure 4: Effect of different concentrations of glucose for production (U/ml) of protease from bacterial strain T3. 1=0.5%, 2=1.0%, 3=1.5%, 4=2.0%, 5=2.5% and 6=3.0%. Where 1, 2, 3, 4, 5 and 6 represent the different concentrations of glucose.

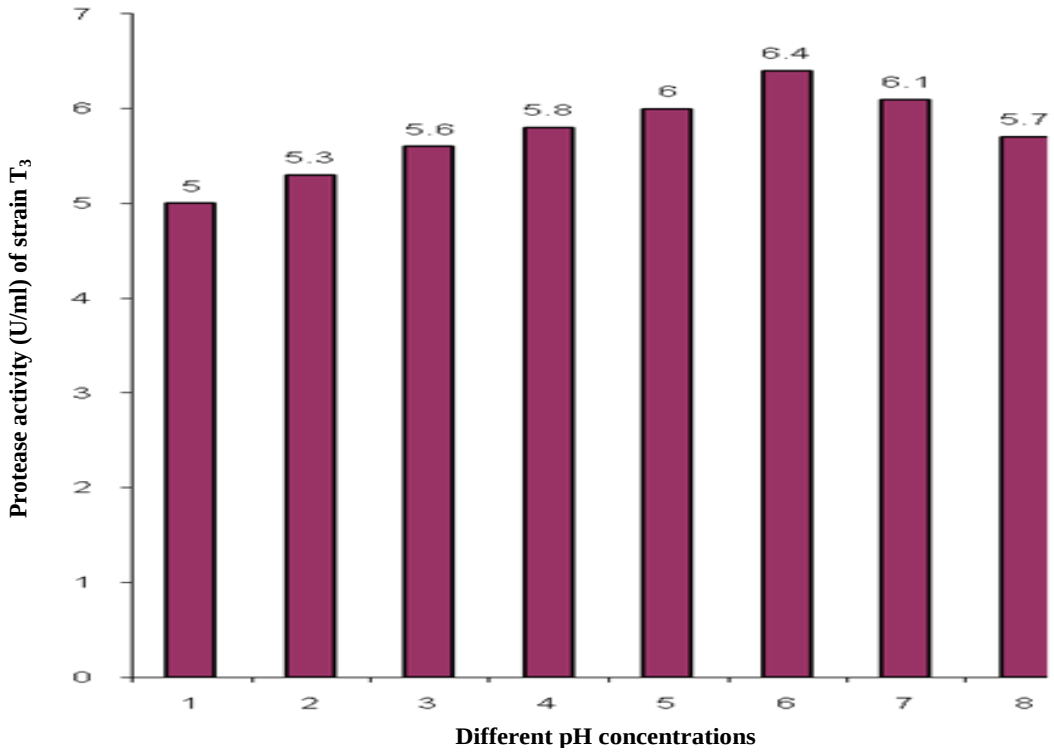

Figure 5: Effect of different pH concentrations for production (U/ml) of protease from bacterial strain T_3. 1=5.5, 2=6.0, 3=6.5, 4=7.0, 5=7.5, 6=8.0, 7=8.5, 8=9.0. Where 1, 2, 3, 4, 5, 6, 7 and 8 represent different pH concentrations.

(Figure 5) respectively. The same procedure was repeated for preparation and incubation of fermentation media under the same condition as earlier described. Thereafter, the activity of protease was checked and compared with each other and the protease production (U/ml) of different pH concentrations later compared. Accordingly, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0% concentration of pH showed 5.0, 5.3, 5.6, 5.8, 6.0, 6.4, 6.1 and 5.7 U/ml of protease production respectively. From the result of the experiment 8.0% concentration of fructose (6.4 U/ml) was declared the best fructose concentration for bacterial strain T_3.

Effect of immobilization on production (U/ml/g support) of partially purified protease from bacterial strain T_3

After the optimization of carbon and nitrogen sources with their optimized concentrations, bacterial strain T_3 showed its highest protease production (6.4 U/ml). Protease production increased (12 U/ml) after partial purification. Different supports Amberlite (XAD 761), Duolite (A568), Pentynyl Dextran (NT4L360) and Lewatit (VPOC 1600) were used for immobilization of partially purified protease. Immobilized protease production was 25 U/ml/g support when partially purified protease was immobilized on Amberlite (XAD 761). Immobilized protease production was 65 U/ml/g support when partially purified protease was immobilized on Lewatit (VPOC 1600). Immobilized protease production was 27 U/ml/g support when partially purified protease was immobilized on Duolite (A568). Immobilized protease production was 418 U/ml/g support when partially purified protease were immobilized on Pentynyl Dextran (NT4L360) (Figure 6).

Increase of protease production (%) after immobilization from bacterial strain T_5, T_3 and H_3

Protease production increased after immobilization. As such, percentage increase of protease production with different immobilizing supports was compared. For bacterial strain T_3, different supports Amberlite (XAD 761), Lewatit (VPOC 1600), Duolite (A568) and Pentynyl Dextran (NT4L360) were shown to be 208.33, 541.66, 225 and 3483.33% more protease production respectively. When partially purified protease was immobilized on Amberlite (XAD 761) the increase of protease production was 208.33% (2.08 fold) for strain T3 and when partially purified protease was immobilized on Duolite (A568), the increase of protease production was 225% (2.25 fold) for strain T_3. When partially purified protease were immobilized on Lewatit (VPOC 1600); the increase of protease production was 541.66% (5.41 fold) for strain T_3 and when partially purified protease was immobilized on

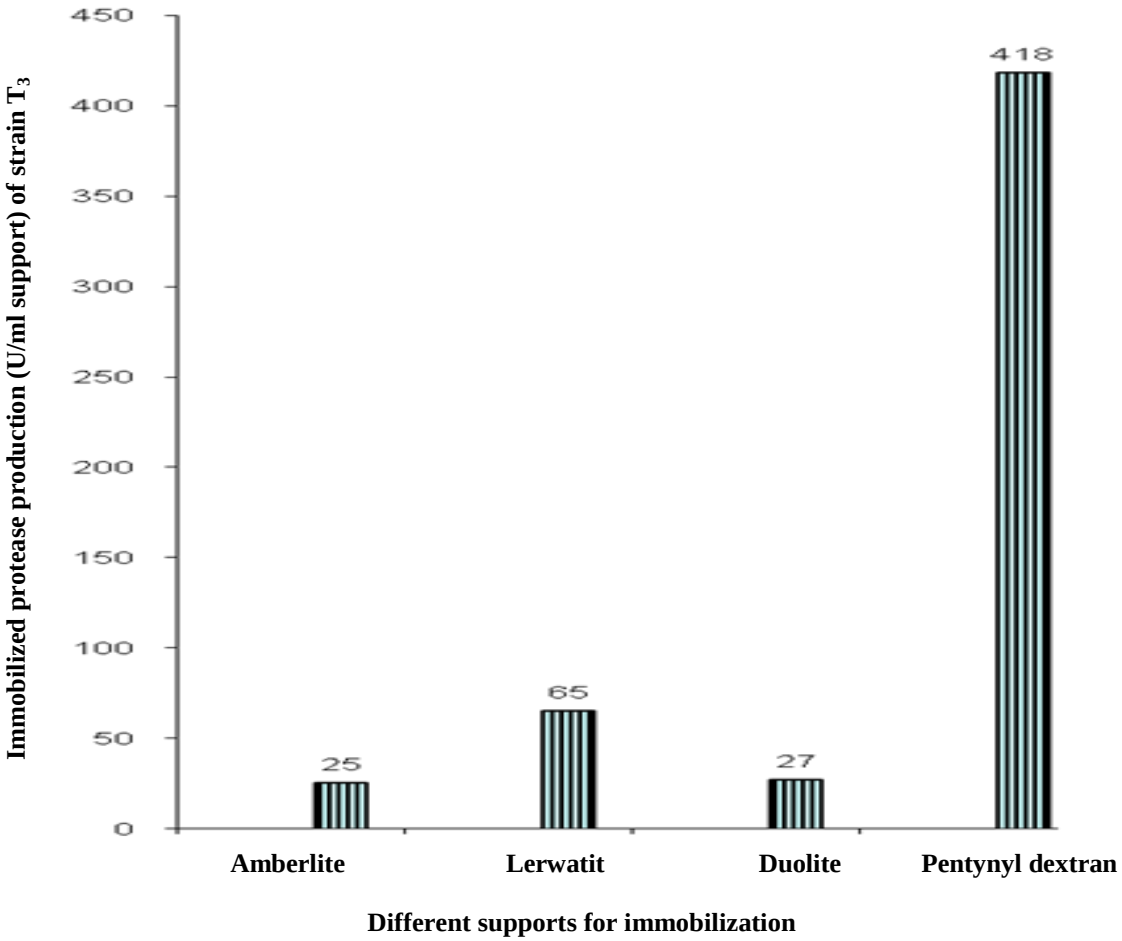

Figure 6: Effect of immobilization on production (U/ml/g support) of partially purified protease from bacterial strain T_3.

Pentynyl Dextran (NT4L360), the increase of protease production became 3483.33% (35 fold) for strain T_3.

DISCUSSION

Gupta et al. (2002) in their study showed that optimization of media components played vital role in increasing the alkaline protease synthesis. According to Haulon et al. (1982), extracellular proteases synthesis was shown to be sensitive to repression by various sources of carbon and nitrogen.

It was investigated by Bomonamdhan (2009) that glucose was the best carbon source for protease production. Same result was also investigated by Sindho et al. (2009) and Shafee (2005) reported that glucose was the best source of carbon for protease synthesis and fructose resulted in being the second best source of carbon for protease synthesis. These results demonstrated that the amount of glucose in the medium has considerable effect on the synthesis of the clotting enzyme and, in lower initial glucose concentrations; enzyme activity was higher as reported by De Lamia et al. (2008). According to Beyenal et al. (1999) and Seker and Beyenal (1999), a higher glucose concentration was a preponderant factor in the inhibition of protease activity, as it stimulated biomass production rather than rennin synthesis.

It was reported by Sonnleitner (1983) that soybean meal proved to be the best source of nitrogen for protease synthesis. Similar result was also investigated by Kalaiarasi and Sunitha (2009). However, production medium enriched with soyabean meal was reported as the best source of nitrogen for protease synthesis as investigated by Sinha and Satyanarayana (1999). Therefore, alkaline extracted soybean was used as the most suitable source of nitrogen for maximal enzyme synthesis. This result is in agreement with that obtained by Mabrouk et al. (1999). It was also investigated by Ahmed et al. (2008) that soybean meal was the best source of nitrogen for protease synthesis.

Investigations by Sindho et al. (2009) showed that fermentation media with pH 8 was the best for protease synthesis. Similar result was also reported by Patel et al. (2005) from alklalophilic nature and *bacillus* sp. The obtained results are in accordance that of Kumar et al. (2002) who reported that protease production was

maximum at pH 7 and 9 for *Bacillus* sp. strain S4 and *Pseudomonas* sp. strains S_{22} respectively. Alkaline protease production by *Streptomyces avermectinus* NRRL B-8165 was considerably influenced by the initial pH values as reported by Ahmed et al. (2008). It was noticed that pH 7.5 was the most favorable for alkaline protease production. Above and below initial pH values showed a gradual decrease in protease production. This result is similar to those reported by Banik and Prakash (2004) who synthesized alkaline protease maximally at pH 7.5 after 72 h of incubation from *Bacillus cereus*.

Immobilization of protease was practiced by several authors (Kumari et al., 2009; Rao et al., 2008). Immobilization is a technique for protecting cells from shear forces. Many different strategies for immobilizing cells were proposed by Beshay et al. (2002) and Abd-El-Haleem et al. (2003). When partially purified proteases were immobilized on Amberlite (XAD 761) the increase of protease production was 208.33% (2.08 fold) for strain T_3. Immobilized protease production was 25 U/ml/g support when partially purified protease was immobilized on Amberlite (XAD 761) for strain T_3. It was reported by Abdle- Naby et al. (1998) that immobilized protease production was 28.09 U/g support (15%) when *Bacillus mycoides* protease was immobilized on Amberlite IR-120.

When partially purified protease was immobilized on Pentynyl Dextran (NT4L360), the increase of protease production was 3483.33% (35 fold) for strain T_3. It was investigated by Yamagata et al. (1994) that immobilized protease production was 56% when protease from *Bacillus sp*. was immobilized on dextran.

Immobilized protease production was 418 U/ml/g support when partially purified protease was immobilized on Pentynyl Dextran (NT4L360) for strain T_3. It was reported by Ahmed et al. (2008) that immobilized protease production was 37.77 U/g support (42.36%) when protease from *Streptomyces avermectinus* NRRL B-8165 was immobilized by physical adsorption on sponge. It was reported by Ahmed et al. (2007) that immobilized protease production was 70.5% when protease from *Bacillus licheniformis* was immobilized by physical adsorption on loofa (as a new carrier). It was investigated by Beshay (2003) that immobilized protease production was 176.8% when protease from *Teredino bacterturnirae* was immobilized by entrapment in calcium alginate gel. The high stability of the immobilized enzyme could be due to the diminished autolysis of the enzyme fixed to the carrier. The second possible explanation may be related to the rigidity of the conformation of the enzyme molecules resulting from binding to the carrier (Afaq and Iqbal, 2001).

Conclusion

The present research work deal with immobilization of bacterial protease and its potential applications. Protease producing bacteria were isolated from different soil samples. By using sub-merged fermentation technique, glucose (1.5%) and soybean meal (2%) was found best carbon and nitrogen source for protease production from bacterial strain T_3. Protease production from strain T_3 was showed its best activity at pH 8. Partial purification was also showed its vital role for enhancing protease production. Protease production was maximum (418U/ml/g support) from bacterial strain T_3 when protease was immobilized on Pentynyl Dextran (NT4L360). An increase of 3483.33% (35 fold) protease production was observed after its immobilization through physical adsorption. We may use this immobilized protease for the synthesis of aspartame (artificial sweetener). This immobilized protease with its highest activity may be used in food, pharmaceutical and leather industry.

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