

*Full Length Research Paper*

## Comparison of protease production from newly isolated bacterial strains T<sub>5</sub>, T<sub>3</sub> and H<sub>3</sub> after immobilization

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Received 25 June, 2016; Accepted 19 September, 2016

Protease has gained a very important position in many industries such as food, pharmaceutical, chemical and leather industries. In this research, protease was obtained from bacteria. The bacterial strain was obtained from soil which was collected from different areas of Lahore, Pakistan. Fermentation medium (by using sub-merged fermentation technique) was incubated for 48 h at 37°C temperature and agitation speed of 200 rpm. The protease was partially purified with 70% ammonium sulphate. Four different supports were used for the immobilization of the bacterial protease by physical adsorption method. When partially purified protease was immobilized on Amberlite (XAD 761), its production amount was 371.42% (52 U/ml/g support) with strain T<sub>5</sub>; 208.33% (25 U/ml/g support) with strain T<sub>3</sub> and 342.24% (64 U/ml/g support) with strain H<sub>3</sub>; when it was immobilized on Duolite (A568), its amount of production was 314.28% (44 U/ml/g support) with strain T<sub>5</sub>; 225% (27 U/ml/g support) with strain T<sub>3</sub> and 395.72% (74 U/ml/g support) with strain H<sub>3</sub>; when it was immobilized on Lewatit (VPOC 1600), the amount of production was 450% (63 U/ml/g support) with strain T<sub>5</sub>; 541.66% (65 U/ml/g support) with strain T<sub>3</sub> and 320.85% (60 U/ml/g support) with strain H<sub>3</sub>. But, when it was immobilized on Pentynyl Dextran (NT4L360), its amount of production was 271.42% (38 U/ml/g support) with strain T<sub>5</sub>, 3483.33% (418 U/ml/g support) with strain T<sub>3</sub> and 304.81% (57 U/ml/g support) with strain H<sub>3</sub>. Immobilized protease from bacterial strain T<sub>3</sub> had the highest immobilizing activity of 3483.33% (418 U/ml/g support). This immobilized protease with the highest activity could be used in food, pharmaceutical and leather industries.

**Key words:** Protease, bacterial strain, immobilizing activity.

### INTRODUCTION

Proteases, also called proteinases or peptidases work as molecular knives by cutting long amino acid sequences into fragments, which are necessary for synthesis of other proteins, regulating their size, composition, shape, turnover and ultimate destruction (Seife, 1997). Proteases

have diverse applications in a wide variety of industries like detergent, food, pharmaceutical and leather industries, and can be used for peptide synthesis and the recovery of silver from used X-ray films.

Micro-organisms are excellent sources for synthesis of

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alkaline protease than plants and animals as they have better advantages such as broad biochemical diversity (Mahto and Bose, 2012). Submerged and solid-state fermentations are the techniques for the synthesis of proteases (Sandhya et al., 2005). Submerged fermentation technique is most widely used for protease synthesis and 90% of proteases are synthesized through this technique (Olker et al., 2004).

Enzyme immobilization means an 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). Immobilization of protease is an efficient tool. For many years, it has got many fruitful benefits more than free protease; they include increase in production rate, operational stability, possibility in reuse of protease, and recovery of final product free of protease contamination (Maghsoodi et al., 2013).

Immobilization of enzymes has the ability to change its catalytic activity or thermal stability (Trevan, 1980). Intrinsic activity of immobilized enzyme is being changed after immobilization. Operational stability of enzyme increases due to immobilization of enzyme (Blanco et al., 1989). From biotechnological point of view, immobilized protease is very useful in pharmaceutical and medicinal, food industries; it is used for wastewater treatment and peptide synthesis in brewing and baking industry, detergent industry, leather and textile industry (Maghsoodi et al., 2013).

In physical adsorption, enzymes and matrixes are linked together through hydrogen bonding, van der Waals forces, or hydrophobic interactions (Bahulekar et al., 1991). This is a simple, cheap and easy method of preparing an immobilized enzyme. It is a carrier bound method of immobilization which is reversible. The enzyme's catalytic activity is preserved by physical adsorption (Brady and Jordan, 2009). Due to the increased loading of enzyme after immobilization, the process improves the operational stability which causes controlled diffusion (Nisha et al., 2012).

## MATERIALS AND METHODS

### Inoculum preparation

Nutrient broth (0.8 g) was weighed and then dissolved in 50 ml of distilled water. Water was added continuously 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 transferred to inoculum flask and then kept in a shaking incubator with 200 rpm agitation speed at 37°C temperature for 24 h.

### Fermentation medium

Submerged fermentation technique was used for the protease synthesis. 2 g of soybean meal, 1.5 g of glucose, 2 g of peptone, 0.1 g  $(\text{NH}_4)_2\text{SO}_4$ , 0.1 g  $\text{KH}_2\text{PO}_4$  and 0.5 g sodium carbonate were

weighed and then dissolved in 50 ml of distilled water. Water was added continuously in the solution until it was marked with 100 ml of that solution. The cotton plugged flasks were 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. Then centrifugation test was done with the sample in a tube at 6000 rpm for 10 min.

### Assay of protease

The activity of the protease was checked through a special procedure introduced by McDonald and Chen (1965). 1.0 ml of protease extract (which was obtained after centrifugation at 6000 rpm for 10 min) was poured in the test tube and 4.0 ml of 1.0% w/v casein was also added in the same test tube. The test tube was placed in an incubator for one hour at 37°C temperature. After that, 5 ml of 5% w/v trichloroacetic acid (TCA) was added to the test tube. After 30 min, the test tube sample was passed under the process of centrifugation (6000 rpm) for ten minutes. One milliliter of supernatant was mixed with alkaline reagent (5 ml). Then 1 ml of 1N sodium hydroxide was mixed in the test tube. Then the test tube sample was allowed to react with each other for 10 min. Folin and Ciocalteu reagent (0.5 ml) was added; blue color was produced 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)".

The activity of the enzyme is 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.

### Purification and concentration of protease

Purification and concentration was done by sorting out the enzyme through the addition of ammonium sulphate.  $(\text{NH}_4)_2\text{SO}_4$  was continuously mixed with the enzyme at a temperature of 4°C. The addition of  $(\text{NH}_4)_2\text{SO}_4$  was continuous up to 70% of saturation. The solution was kept in cool laboratory (at 4°C) for ten minutes. After 30 min, test tube sample was passed through the process of centrifugation (10000 rpm) 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. Then 10 ml of dissolved pellets were poured in a given dialysis tube and kept in 1000 ml of 0.1 M Tris-HCl buffer solution with continuous stirring for 24 h at 4°C. During this period, the buffer was refreshed 3 to 4 times. The purified and concentrated enzyme was used for immobilization studies (Lowry et al., 1971).

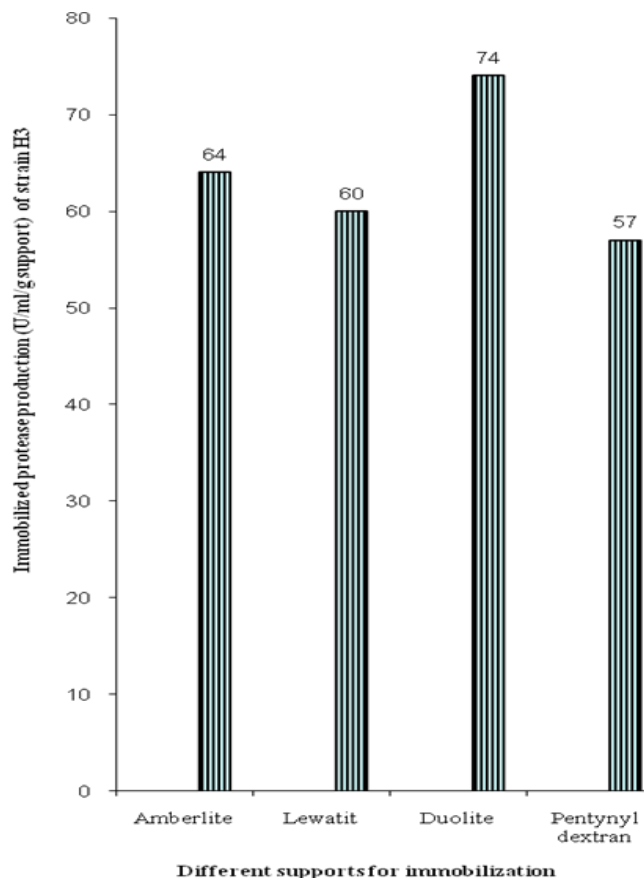
### Immobilizing supports

Different supports for immobilization were utilized like:

1. Lewatit VPOC 1600 (Lanxess- Germany) (Tu Braunschweig, Germany),
2. Duolite A568 (Rohm and Hass- France) (Tu Braunschweig, Germany),
3. Amberlite XAD 761 (Rohm and Hass- France) (Tu Braunschweig, Germany),
4. Pentynyl Dextran (NT4L360) Insitute Fur Lebensmittel Chemie (Tu Braunschweig, Germany),

### Physical adsorption (Immobilization technique)

Enzyme was immobilized on different supports. 0.5 g of each

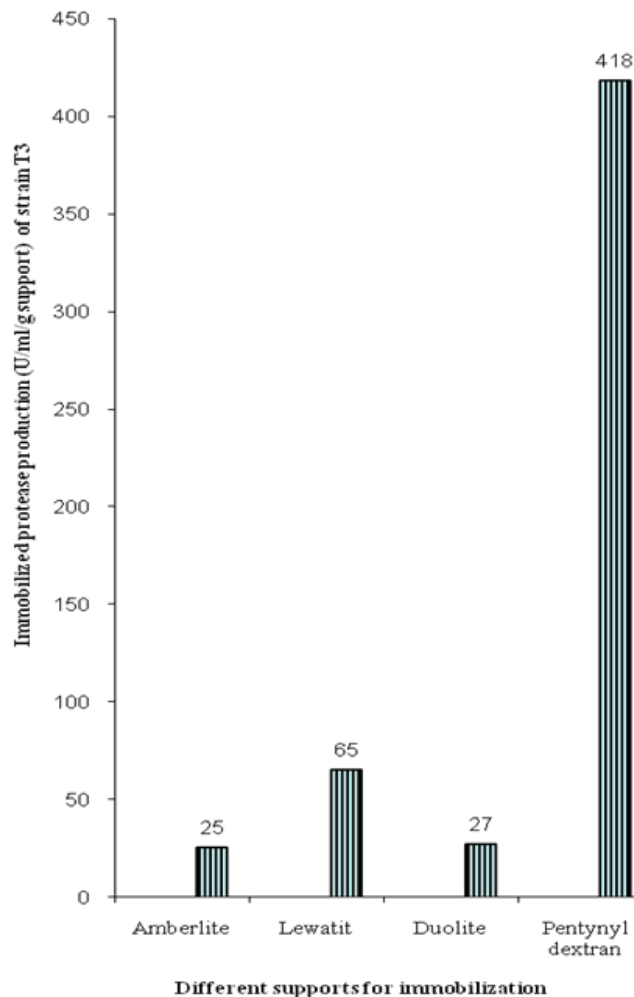


**Figure 1.** Effect of immobilization for production (U/ml/g support) of protease from partially purified bacterial strain H<sub>3</sub>.

support was stirred with 5 ml of partially purified protease and kept in a shaking water bath at 100 rpm speed and 37°C for 60 min. Then it was centrifuged at 6000 rpm for 10 min. Thereafter, the supernatant was used for protease activity (Minovska et al., 2005).

## RESULTS AND DISCUSSION

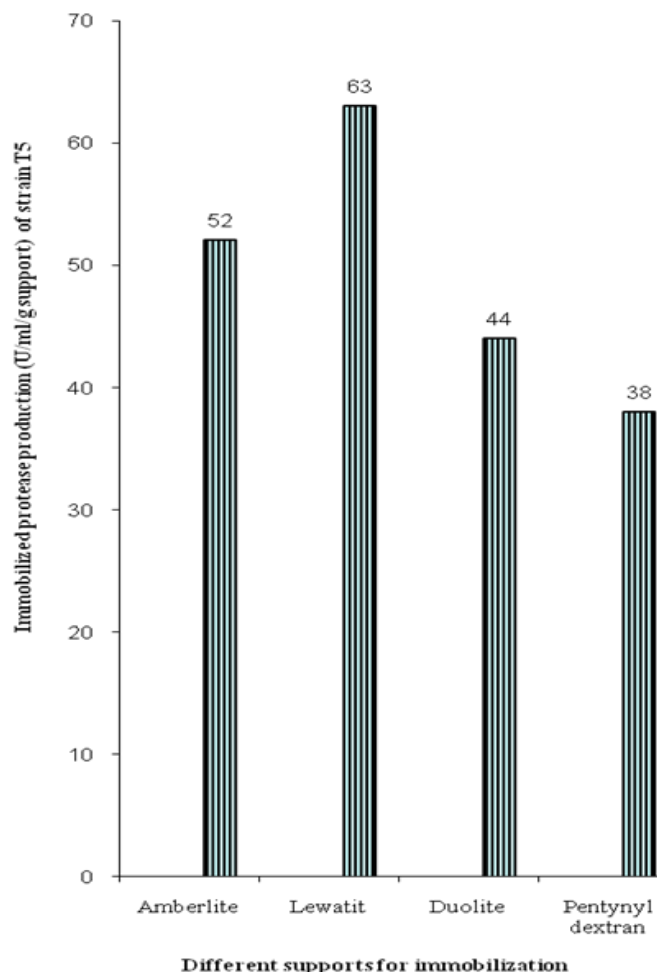
In Figure 1, the protease activity for bacterial strain H<sub>3</sub> was increased from 12.6 to 18.7 U/ml and then its activity further increased after immobilization. Immobilized protease production was 64, 60, 74 and 57 U/ml/g support when partially purified protease was immobilized on Amberlite (XAD 761), Lewatit (VPOC 1600), Duolite (A568) and Pentynyl Dextran (NT4L360) respectively. In Figure 2, protease activity for bacterial strain T<sub>3</sub> increased from 6.4 to 12 U/ml and then its activity further increased after immobilization. Immobilized protease production was 25, 65, 27 and 418 U/ml/g support when partially purified protease was immobilized on Amberlite (XAD 761), Lewatit (VPOC 1600), Duolite (A568) and Pentynyl Dextran (NT4L360) respectively. In Figure 3, protease activity for bacterial strain T<sub>5</sub> increased from 10.7 to 14 U/ml and then its activity further increased after immobilization. Immobilized protease production was 52,



**Figure 2.** Effect of immobilization on production (U/ml/g support) of partially purified protease from bacterial strain T<sub>3</sub>.

63, 44 and 38 U/ml/g support when partially purified protease was immobilized on Amberlite (XAD 761), Lewatit (VPOC 1600), Duolite (A568) and Pentynyl Dextran (NT4L360) respectively

In Figure 4, protease activity increased after immobilization. So the percentage increase of protease activity with different immobilizing supports was compared. For bacterial strain T<sub>5</sub>, four different supports such as Amberlite (XAD 761), Lewatit (VPOC 1600), Duolite (A568) and Pentynyl Dextran (NT4L360) of 371.42, 450, 314.28 and 271.42% increased protease production respectively. For bacterial strain T<sub>3</sub>, four different supports such as Amberlite (XAD 761), Lewatit (VPOC 1600), Duolite (A568) and Pentynyl Dextran (NT4L360) of 208.33, 541.66, 225 and 3483.33% increased protease production respectively. For bacterial strain H<sub>3</sub>, four different supports such as Amberlite (XAD 761), Lewatit (VPOC 1600), Duolite (A568) and Pentynyl Dextran (NT4L360) of 342.24, 320.85, 395.72 and 304.81% increased protease production respectively.



**Figure 3.** Effect of immobilization on production (U/ml/g support) of partially purified protease from bacterial strain T<sub>5</sub>.

## DISCUSSION

When the partially purified proteases were immobilized on Amberlite (XAD 761), increase in protease production was 371.42% (3.71 fold) with strain T<sub>5</sub>, 208.33% (2.08 fold) with strain T<sub>3</sub> and 342.24% (3.42 fold) with strain H<sub>3</sub>. Thus for Amberlite (XAD 761), bacterial strain T<sub>5</sub> had shown its highest activity with 3.71 fold. Ibrahim et al. (2016), using three different matrixes (HCMSS-non, HCMSS-NH2 and HCMSS-C2H5) for immobilization of protease through physical adsorption, found 40, 42 and 16.3% activity yield with respect to the above supports. Immobilization yield was 44.6, 47.3 and 19.3% for matrixes like HCMSS-non, HCMSS-NH2 and HCMSS, respectively. Ahmed et al. (2007) reported that immobilized protease production was 70.5% when protease from *Bacillus licheniformis* was immobilized by physical adsorption on loofa (as a new carrier).

When partially purified protease were immobilized on Lewatit (VPOC 1600), then increase in protease

production was 450% (4.5 fold) with strain T<sub>5</sub>, 541.66% (5.41 fold) with strain T<sub>3</sub> and 320.85% (3.20 fold) with strain H<sub>3</sub>. Thus for Lewatit (VPOC 1600), bacterial strain T<sub>3</sub> had shown its highest activity with 5.41 fold. When purified protease from *B. subtilis* M-11 was immobilized on PS membrane containing Si-APTS by physical adsorption the specific activity of protease was 175.43 EU/mg and protease activity was increased up to 15.8 fold with 1.5% immobilization yield (Selmihan et al., 2015).

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). Upon immobilization of the purified protease on Duolite (A568), increase in protease production was 314.28% (3.14 fold) with strain T<sub>5</sub>, 225% (2.25 fold) with strain T<sub>3</sub> and 395.72% (3.95 fold) with strain H<sub>3</sub>. Thus for Duolite (A568), bacterial strain H<sub>3</sub> highest activity was 3.95 fold.

Immobilized protease attached onto the activated HCMSS-NH2 nanospheres showed the highest immobilization yield (75.6%) and loading capacity (88.1 µg protein/mg carrier). In addition, the immobilized protease affinity to the substrate was increased up to 1.5-fold (Ibrahim et al., 2016). When partially purified protease was immobilized on Pentynyl Dextran (NT4L360), increase in protease production was 271.42% (2.71 fold) for strain T<sub>5</sub>, 3483.33% (35 fold) for strain T<sub>3</sub> and 304.81% (3.42 fold) for strain H<sub>3</sub>. Thus for Pentynyl Dextran (NT4L360), bacterial strain T<sub>3</sub> had shown its highest activity with 35 fold.

## Conclusion

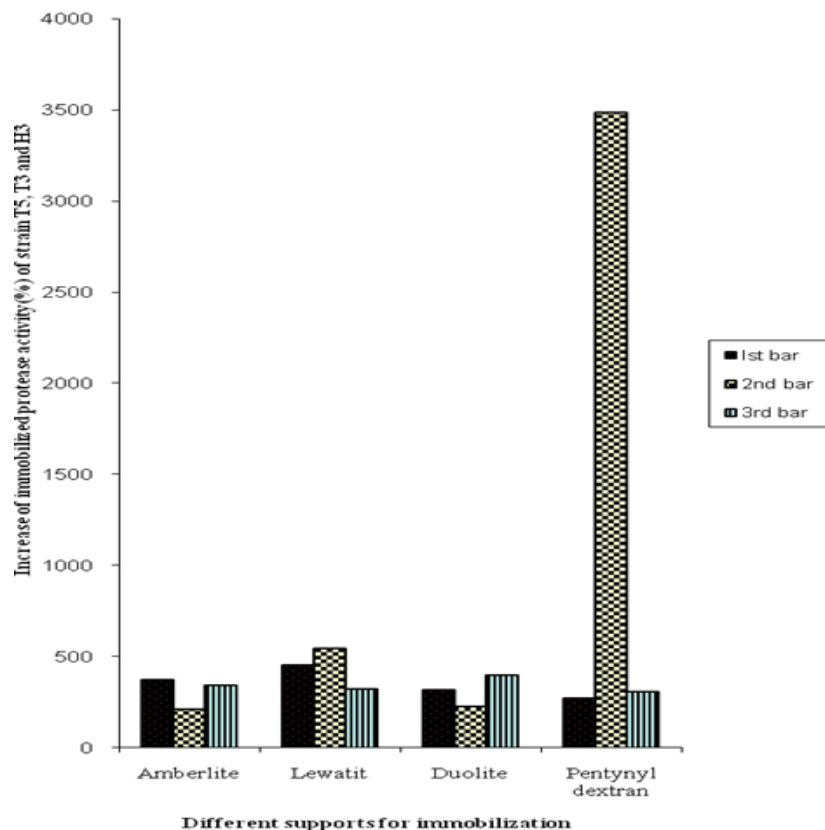
The present research work compares the proteases produced from newly isolated bacterial strains, T<sub>5</sub>, T<sub>3</sub> and H<sub>3</sub> after immobilization. Maximum protease production was obtained (418 U/ml/g support) from bacterial strain T<sub>3</sub> after immobilization on Pentynyl Dextran (NT4L360). Finally, increase of immobilized protease activity was approximately 35 fold through physical adsorption method. We may use this immobilized protease for the synthesis of aspartame (artificial sweetener). In general, this immobilized protease with its highest activity could be used in food, pharmaceutical and leather industries.

## Conflict of Interests

The authors have not declared any conflict of interests.

## ACKNOWLEDGEMENTS

The author is grateful to his father, Mr. Ilyas Javaid and mother Mrs. Tehmina Ilyas for his inherited courage and determination.



**Figure 4.** Increase of protease production (%) after immobilization from bacterial strain T<sub>5</sub>, T<sub>3</sub> and H<sub>3</sub>. 1<sup>st</sup> bar = Production (%) of immobilized protease from strain T<sub>5</sub>; 2<sup>nd</sup> bar = Production (%) of immobilized protease from strain T<sub>3</sub>; 3<sup>rd</sup> bar = Production (%) of immobilized protease from strain H<sub>3</sub>.

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