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Optimized production of extracellular proteases by *Bacillus subtilis* from degraded abattoir waste

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ABSTRACT

Proteases are ubiquitous in occurrence and are found in all living organisms. These are essential for cell growth and differentiation. The extracellular proteases are of a high commercial value and find multiple applications in various industrial sectors. The present study describes the screening of protease producing bacteria from a hitherto unexplored source i.e. degraded waste from abattoir. Three isolates were found namely yellow, white and orange coloured bacteria. Amongst them, white colored colony was found to be more suitable for protease production. The morphological, cultural, biochemical and 16S rRNA confirmed that the isolate was *Bacillus subtilis*. Physical and chemical parameters were optimized for maximum protease production and optimum temperature and pH was found to be 40°C at pH 14. Glucose as a carbon source and yeast extract as a nitrogen source further stimulated the production process giving maximum protease activity to be 20.74 U/ml and 20.67 U/ml. The applications of protease in detergent and solvent industry were tested and it was revealed that the purified enzyme can be used as an additive in detergent industry.

Key words: *Bacillus subtilis*, protease, textiles, abattoir waste, enzyme activity, optimization, detergent industry

Introduction

The enzymes are considered as “green chemicals” due to their ecofriendly nature. Also, they possess wide range of applications ranging from industrial sector to house-hold products (Rai & Mukherjee, 2010). More than 3000 different enzymes have been identified and many of them are being used in biotechnological and industrial applications (Suganthi et al., 2013).

Proteolytic enzymes are found in all living organisms, and are essential for cell growth and differentiation. There is renewed interest in the study of proteolytic enzymes, mainly due to the recognition that these enzymes not only play an important role in the cellular metabolic processes, but have also gained considerable attention in the industrial community (Gupta et al., 2005). The demand for proteolytic enzymes having appropriate specificity and stability to pH, temperature, metal ions, surfactants and organic solvent is now rising, stimulating the search for more new enzyme sources (Kamoun et al., 2008).

Proteases (E.C.3.4.21.14) are enzymes that break peptide bonds between amino acids of proteins (Sathyavathan & Kavitha, 2013). Proteases represent one of the three largest groups of industrial enzymes and account for about 60% total worldwide sale of enzymes (Rao et al., 1998). Several microbial strains including fungi such as *Aspergillus flavus*, *Aspergillus miller*, *Aspergillus niger* and *Penicillium griseofulvin* and different bacteria namely *Bacillus licheniformis*, *Bacillus firmus*, *Bacillus alcalo* *Bacillus subtilis* and *Bacillus thuringiensis* etc. have been reported to produce proteases. Among these, *Bacillus* genus gained importance at industrial scales (Kamoun et al., 2008).

Proteases are important components of biopharmaceutical products such as contact lens cleaners and enzymatic debriders (Anwar & Saleemuddin, 2000). Proteolytic enzymes support the natural healing process in local management of skin ulceration by efficient removal of necrotic material (Sjodahl et al., 2002). Proteases catalyze or hydrolyze protein (Schallmey et al., 2004) and therefore play a vital role in various industrial applications (Li et al., 2004;

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Nijland & Kuipers, 2008). Hyperactive strains are being sought for use in different industries (Fu *et al.*, 2007) such as food, detergent, dehairing and bating of hides, textile, wool quality improvement, in tanning industry, pharmaceutical, leather processing (Prakasham *et al.*, 2005), brewing, meat, photographic (Anwar & Saleemuddin, 1998).

Many researchers are moving towards exploring new sources for producing various enzymes. Conversion of wastes into useful biomass by microorganisms and their enzymes is a new trend, and new protease-producing microorganisms and perfected fermentation technology are needed to meet the ever-growing demand for this enzyme (Rathakrishnan *et al.*, 2012).

Waste products of meat, poultry and fish processing industries can supply a large amount of protein rich material for bioconversion to recoverable products (Dalev, 1994). Research has been done on many other related sources for protease production; a few to mention are, slaughter house soil (Gaustevora *et al.*, 2005), dairy industrial effluent, market waste, sewage waste (Vasantha & Subramanian, 2012).

The majority of the waste, in the meat industry is produced during slaughtering. Slaughter house waste consists of the portion of a slaughtered animal that cannot be sold as meat or used in meat-products. Such waste includes bones, tendons, skin and the contents of the gastro-intestinal tract, blood and internal organs. These vary with each type of animal (Udandi *et al.*, 2009; Jayathilakan *et al.*, 2012). These wastes are most of the time dumped to degrade. We have selected such degraded waste for our study, as it is available freely and in large amount. Moreover, it is inexpensive source, as it is a waste one.

Further physicochemical parameters for maximum protease production from *B. subtilis* were optimized followed by purification and characterization studies.

Materials and Methods

Collection of samples

The experimental sample of degraded abattoir waste was collected in a sterile container from local meat shop, Wadala, Mumbai, India, as per the microbiological procedures and was shifted to the laboratory for further analysis.

Screening of bacteria for protease production

The loopful culture of abattoir waste sample was streaked on nutrient agar plate. It was serially diluted and streaked on

gelatin agar plates. The plates were incubated for 24 h at 37°C and enzyme activity was observed. Colonies producing zone of hydrolysis around them were selected as protease producing bacteria. Zone of hydrolysis was analyzed by flooding gelatin agar plates with 0.25% tannic acid solution for 15-20 min.

Identification of protease producing bacteria

Gram's staining

A drop of sterile saline water was placed on the microscope slide. A light suspension of the test culture was made with normal saline and heat fixed. The slide was allowed to cool and flooded with crystal violet for 60 sec and then washed with distilled water. It was then flooded with Gram's iodine for 60 sec, washed with water and excess water was drained off. The stain was then decolorized with acetone which was washed off immediately. Counterstaining was done with safranin for 2 min. The slide was then washed, air dried and observed under microscope.

Biochemical test

Morphological and physiological characteristic of bacterial isolate were studied according to method described in Bergey's Manual of Determinative Bacteriology.

Taxonomic identification

Based on the morphological and biochemical tests, the bacterial isolate was primarily identified by Gram staining. The taxonomic identification was carried out at National Centre for Cell Sciences (NCCS), University of Pune Campus, using 16S rRNA method.

Protease assay

Protease activity was determined by the standard Folin-Lowery assay with some modifications. The reaction mixture containing 5 ml of casein (prepared in 50 mM of Tris buffer, pH 8.0) and 1 ml of the enzyme solution was incubated for 30 min. The reaction was stopped by adding 5 ml of 0.11 M trichloroacetic acid solution. After 30 min, the mixture was filtered and 2 ml of the filtrate was added to 5 ml of 0.5 M sodium carbonate along with 1 ml of Folin - Ciocalteu's phenol reagent and this was kept for 30 min at 37°C (Tsuchida *et al.*, 1986). The optical densities of the solutions were observed against the blank sample at 660 nm using UV-Visible Spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per ml per min under assay conditions (Lowry *et al.*, 1951).

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The isolate was grown in modified Gelatin yeast extract broth containing 1% gelatin, 1% glucose, 0.2% yeast extract and 0.3% K₂HPO₄. It was incubated at 40°C up to 32 h in an orbital shaker at 150 rpm. The contents were then centrifuged at 5000 rpm at 4°C for 20 min and protease activity was checked by the Folin-Lowery assay method in the cell free extract (Josephine *et al.*, 2012).

Effect of temperature on protease production

The effect of temperature on the enzyme activity was determined in the range of 20 to 80°C for 24 h in an orbital shaker at 150 rpm. The contents were centrifuged at 5000 rpm at 4°C for 20 min and protease activity was checked in the cell free extract.

Effect of pH on the protease production

Effect of pH on the protease production was studied by adjusting the culture media pH at 3, 5, 7, 9, 10, 11, 12, 13 and 14 prior to sterilization. It was incubated at 40°C for 24 h in an orbital shaker at 150 rpm. The contents were centrifuged at 5000 rpm at 4°C for 20 min and protease activity was checked in the cell free extract.

Effect of carbon sources on the protease production

To find the optimum carbon source for enzyme production, 1% of six carbon sources each viz. Sucrose, fructose, glucose, cellulose, lactose and starch were selected and added to gelatin yeast extract broth. The organism was inoculated and incubated for 24 h at 40°C and the enzyme activity was assayed in the culture supernatant.

Effect of nitrogen sources on enzyme production

To optimize the nitrogen source for maximum enzyme production, five different nitrogen sources each of 1% viz. Ammonium nitrate, ammonium chloride, ammonium sulphate, yeast extract, potassium nitrate, sodium nitrate were added to nutrient broth and the organism was inoculated and incubated for 24 h. The enzyme activity was assayed in the culture supernatant.

Characterization***Enzyme purification***

For enzyme purification a two-step purification protocol was followed. The crude enzyme sample was saturated up to 18% using ammonium sulfate and kept overnight at 4°C. The

saturated sample was centrifuged at 6000 rpm for 20 min. The precipitate was suspended in 20 mM Tris buffer having pH 7.0 and was dialyzed against the same buffer to carry out desalting. For further purification of the enzyme, a DEAE cellulose column (1.4×5 cm) was prepared and equilibrated with Tris buffer. Dialyzed enzyme (10% of column's height) was applied to the column and eluted with a linear gradient of NaCl (0.1 M) in Tris-HCl buffer, pH 7.0, at a flow rate of 0.5 ml/min. Protein content of fractions was measured using spectrophotometer at 280 nm and protease activity was assayed by the method reported earlier. The fractions showing peaks for protease activity were pooled together and used as a purified enzyme. This enzyme was used further for characterization studies.

Effect of pH on the activity and stability of the protease

The optimum pH of the enzyme was studied over a pH range of 3–11. Casein (0.65 %) was used as substrate in acidic and alkaline pH respectively. Phthalate-HCl buffer (pH 3), acetate buffer (pH 5), phosphate buffer (pH 7), Tris-HCl buffer (pH 9), and carbonate- biocarbonate buffer (pH 11) were used to determine the enzyme activity. The pH of the reaction mixture was adjusted with the above mentioned buffers at 0.2 M concentration and the enzyme activity was measured by Folin-Lowery assay method. The stability of the enzyme was studied by pre-incubating the enzyme for 60 min at 37°C with each buffer. The assay was then carried out at pH values ranged from 3 to 11 using buffered substrate with pre-incubated enzyme to determine the stability of enzyme.

Effect of temperature on the activity and stability of the protease

The optimum temperature of the enzyme was studied by incubating the reaction mixture over the temperature range of 20–80°C and the enzyme activity was measured by casein assay method. The thermal stability of the enzyme was studied by pre-incubating the enzyme at different temperatures ranging from 20 to 80°C for 60 min at optimized pH. The casein assay was carried out to determine thermal stability of the enzyme.

Effect of ionic and non-ionic detergents on protease activity

The effect of detergents on enzyme activity was studied using ionic and non-ionic detergents 1% such as Tween 20, Tween 80, Triton X 100, SDS. 1% of the above mentioned detergents were added to the enzyme sample and incubated for 30 min. Enzyme activity was assayed by Folin-Lowery method.

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Effect of polar and non-polar solvents on protease activity

The effect of solvents on enzyme activity was studied by incubating the culture supernatant with polar and non-polar solvents 1% such as acetone, benzene, chloroform, hexane and toluene. Enzyme activity was assayed by Folin-Lowery method.

Results and Discussion

In the present study, bacterial culture was isolated from degraded abattoir waste by serial dilution technique and plated on gelatin nutrient agar.

Screening and identification of protease producing organisms

Three colonies were noted on gelatin nutrient agar plates and a clear zone around one colony after 24 h of incubation at 37°C was taken and used for further studies. The organism was identified as belonging to the genus *Bacillus* (Figure 1).



Figure 1. Gram positive, rod shape *Bacillus* sp.

Morphological and physiological characteristics of the bacterial isolate were investigated according to the method described in Bergey's Manual of Determinative Bacteriology. It was identified as a member of *Bacillus* species. The colonial appearance of the *Bacillus* sp. was off white with 6 mm diameter in gelatin nutrient agar plate. The morphological characteristics and the biochemical tests of the *Bacillus* sp. were summarized in Tables 1 and 2. The *Bacillus* sp. was found as Gram positive, aerobic, catalase and oxidase- positive, spore forming and rod shape bacterium (Figure 1).

Table 1. Morphological characteristics of *Bacillus* sp.

Cellular morphology	Results
Colony size	Medium
Colony shape	Circular
Colony colour	Off- white
Margin	Entire
Elevation	Flat
Consistency	Soft
Opacity	Opaque
Endospore	Positive
Position	Central

16S rRNA taxonomical identification

The taxonomical identification by using 16S rRNA was carried out at National Center for Cell Science (NCCS), Pune. It was observed that, the identified organism belongs to genus *Bacillus* and the proteolytic strain belongs to *Bacillus subtilis*.

Effect of the fermentation period on the enzyme production

The effect of fermentation period on the production of protease under various time intervals like 1, 2, 3, 4, 5, 8, 16, 20, 24, 28, 32 h was studied and the maximum enzyme activity was obtained at 24 h (Figure 2).

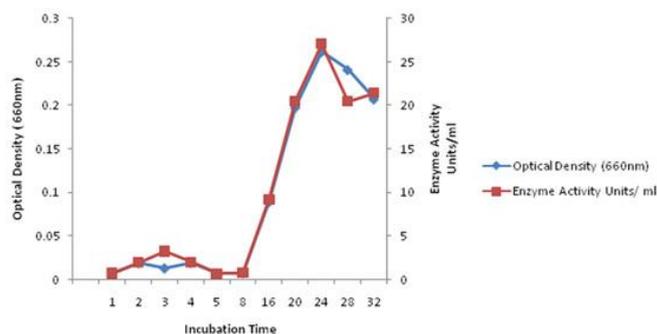


Figure 2. Effect of the incubation time on the protease activity and optical density.

In the present study, highest protease activity of 27.07 U.ml⁻¹ was seen at 24 h of fermentation period. Other investigators reported that both *B. anthracis*, S-44 and *B. cereus* var. *mycooides*, S-98 exhibited their maximum ability to biosynthesize proteases within 24 h incubation period since the productivity reached up to 126.09 U.ml⁻¹ for *B. anthracis*, S-44 corresponding to 240.45 U.ml⁻¹ for *B. cereus* var. *mycooides*, S-98 respectively. Gaustevora *et al.* (2005) found that a high level of extracellular thermostable protease activity was observed after 24 h incubation period.

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Table 2. Biochemical test of *Bacillus* sp.

Name of test	Result
Oxidase	+
Urease	+
Gelatinase	+
Malonate	+
Voges –Proskeur test	-
Citrate Utilization	+
ONPG	-
Nitrate reduction	+
Catalase	+
Arginine	-
Sucrose	+
Starch hydrolysis	+
Lysine decarboxylase	+
Caseinase	+
Reduction of nitrate to nitrite	+
Indole production	-
Arabinose	+
Fructose	+
Galactose	+
Glucose	+
Glycerol	+
Lactose	+
Maltose	+
Mannitol	+
Mannose	-
Voges–Proskauer test	-
Xylose	+

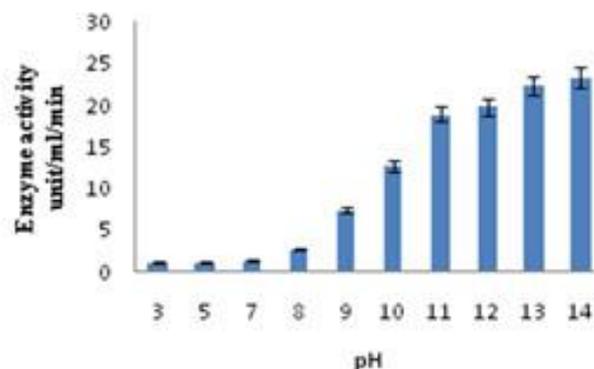
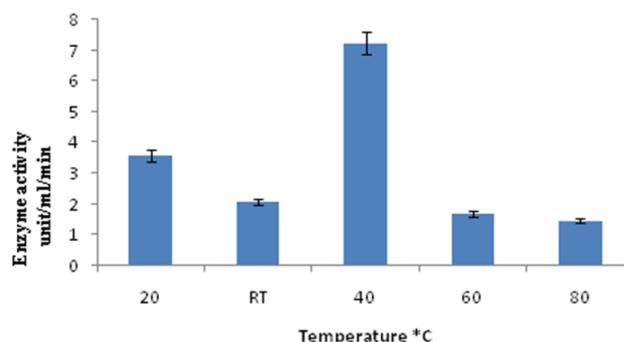
Effect of pH on the enzyme production

The pH variation brought drastic changes in the production of protease. The production medium was adjusted to different pH ranges such as 3, 5, 7, 8, 9, 10, 11, 12, 13, 14 and these flasks were incubated for 24 h. The maximum enzyme activity (23.34 U.ml⁻¹) was seen at pH 14 (Figure 3). Nilegaonkar et al. (2007) reported that the dehairing of goat by using protease of *B. cereus* MCM B-326 had an optimum pH of 9 and (Kumar et al., 1999), reported that optimal pH values for alkaline proteases from *Bacillus* sp. and *B. licheniformis* were in the range of pH 12.0 - 13.0.

Effect of the temperature on the enzyme production

The production medium was applied for various temperature levels at 20 to 80°C. The maximum enzyme yield obtained at 40°C and the maximum enzyme activity was found as 7.24 U.ml⁻¹ (Figure 4). It was reported that 40°C was

the optimum temperature for the protease production from *B. subtilis* (Safe & Raouf, 2004). The activity decreases as the temperature is raised to 60°C, and further decrease was observed at 80°C. Similar trends have been reported where the optimum temperature stability for the protease production from *B. laterosporu* was observed at 40°C (Usharani & Muthuraj, 2009).

**Figure 3.** Effect of pH on the protease activity.**Figure 4.** Effect of the temperature on the protease activity.**Effect of carbon supplement**

In the production medium, various carbon supplements were added. The results indicate that the maximum enzyme production was enhanced by addition of carbon enriched supplements like sucrose, fructose, glucose, cellulose, lactose, and starch. Among these supplements, maximum enzyme activity (20.76 U.ml⁻¹) was obtained when glucose was used (Figure 5). Similar findings were reported by (Sen & Satyanarayana, 1993; Pastor et al., 2001; Santhi, 2014), where glucose and starch gave maximum protease

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production, respectively. These results are in good accordance with previous studies showing maximum protease production when glucose was used as carbon source in the culture broth.

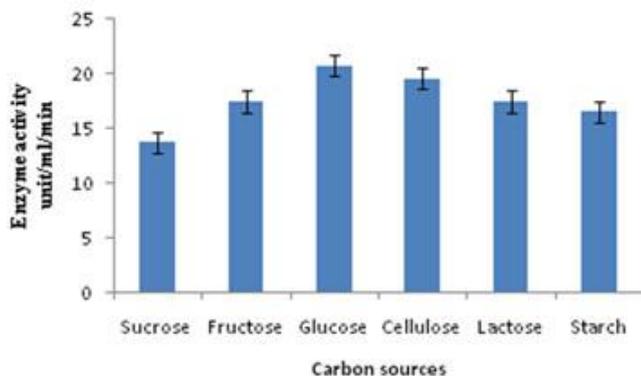


Figure 5 Effect of the carbon source on the protease activity.

Effect of nitrogen supplement

In the production medium, various nitrogen supplements were added. The results indicate that the maximum enzyme production was enhanced by addition of nitrogen enriched supplements like ammonium nitrate, ammonium chloride, ammonium sulphate, yeast extract, potassium nitrate, and sodium nitrate. Among these supplements, maximum enzyme activity ($20.65 \text{ U}\cdot\text{ml}^{-1}$) was obtained when yeast extract was used (Figure 6). Several studies also suggest that yeast extract is a suitable nitrogen source, which stimulates protease production by using various microbial species (Phadatare et al., 1993; Ashour et al., 1996). The production of protease probably was enhanced due to the high protein and amino acid components in the yeast extract.

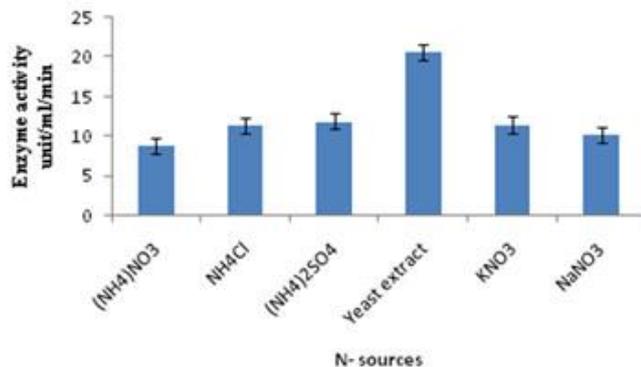


Figure 6. Effect of the nitrogen source on the protease activity.

Growth curve determination of the degraded bacterium *Bacillus sp.*

The protease production was maximum at 24 h of incubation. It can be observed that there was a slow and steady growth of *B. subtilis* in the nutrient medium indicating the lag phase i.e. time required by the organism to adapt itself in the nutrient medium. There was a rise in protease production as well as *Bacillus* growth after 20 h and the protease production maximized at 24 h indicating maximum protease production in the late exponential phase. Beyond 24 h both the protease activity as well as the bacterial growth remained slowly decreases and became constant. Thus, it can be concluded that during fermentation, production of protease from *Bacillus subtilis* starts in the early lag phase and reaches peak in the late log/exponential phase (Figure 2).

Enzyme purification

The extracellular protease produced by *B. subtilis* was purified in two steps by 0-18% ammonium sulphate precipitation followed by anion exchange chromatography on DEAE-cellulose resin. The recovered active fraction from 0-18% ammonium sulphate of culture broth was adsorbed on the DEAE-cellulose matrix. The bound protease was eluted with 0.1 M NaCl (in 10 mM Tris-HCl buffer, pH 7.0). The protease was purified 2.94 fold and about 91.64% of the total activity units were recovered. The specific activity of the purified enzyme was 342.20 U/mg. The purified enzyme could be stored in 10 mM Tris-HCl buffer, pH 7.0, at 4°C for 3 months without any apparent loss of activity. The results of purification of protease from *B.subtilis* are summarized in (Table 3).

Effect of ionic and non-ionic detergents on the protease activity

The enzyme activity was the highest (66.44%) in Triton X-100 and 59.46% for Tween 80 and lowest (51.49%) in Tween 20 and 46.51% in SDS (Table 4). The enzyme was highly stable in presence of detergent like Triton X-100 (Nascimento & Martins, 2006). The extracellular production of the enzyme is compatible with most commercial detergents. These features suggest its application in detergent industry (Naidu & Devi, 2005). In the present study, it was also shown that the protease isolate from *Bacillus* species was able to degrade both ionic and non-ionic detergents.

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Table 3. Purification of protease from *B. subtilis*.

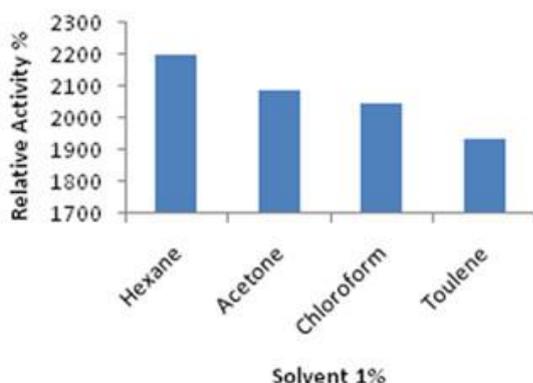
Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification folds	Yield (%)
Culture medium supernatant	407	3.50	116.28	1	100
Ammonium sulfate precipitation	389	2.57	151.36	1.30	95.57
Ion exchange (DEAE cellulose)	373	1.09	342.20	2.94	91.64

Table 4. Effect of ionic and non-ionic detergents on protease activity.

Detergent 1%	Relative activity %
Tween 20	51.49
Tween 80	59.46
Triton 100	66.44
SDS	46.51

Effect of polar and non-polar solvents on the protease activity

Enzymes are usually inactivated or denatured in the presence of polar and non-polar solvents. The protease was found to be tolerant to solvents. The protease was found to be tolerant to solvents. The enzyme exhibited 2200% relative activity in response to hexane, 2088% to acetone, 2044% and 1933% relative activity with chloroform and toluene respectively during the post incubation period (Figure 7). The tolerance exhibited by protease to organic solvents was good; the stability of enzyme in the presence of solvents may be due to replacement of water molecules in the enzyme by organic molecules and thereby stabilizing the structure of the enzyme (Frikha et al., 2003).

**Figure 7.** Effect of polar and non-polar solvents on protease activity.**Conclusion**

The enzymes may serve as a model system and may pave the way for engineering novel ways for eco-friendly industrial applications. Degraded abattoir waste was successfully utilized as a novel source for isolation of *B. subtilis*, which was further used for production of protease enzyme. For maximal yield of enzyme, the optimized fermentation parameters were at 40°C for 24 h and pH 7.0. The nitrogen and carbon sources were also optimized for the same, yeast extract and glucose were found to be the best. Direct application of these highly proteolytic cultures in the rapid production of protease and downstream processing also becomes simpler and cost-effective. This protease have immense applications in textiles, detergent, pharmaceutical, leather, cosmetic, food and paper industries making this study highly significant. Based on the present study, it is concluded that *B. subtilis* has wide scope for the industrial production of protease under submerged fermentation. Even small improvements have been significant for commercial success in biotechnological enzyme production processes.

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