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# Production of protease from biodiesel waste derived semipurified glycerol

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Glycerol generated as the by-product of biodiesel production from vegetable oil or animal fat, has increased the availability of crude glycerol in the market. New strategies need to be developed for the utilization of this abundant carbon source. Using biodiesel waste derived semipurified glycerol as the carbon source, *Bacillus amyloliquefaciens*, *Bacillus megaterium* and *Bacillus cereus* were compared for the production of protease, an industrially important enzyme. Results showed that the medium with 1% semipurified glycerol was an effective carbon source for protease production by these organisms. In addition to glycerol concentration, the parameters such as temperature, pH, incubation period and volume of the inoculum were optimized to increase the protease production. Among the three *Bacillus species* studied, *B. amyloliquefaciens* was found to be the best producer of protease. A maximum protease production of 543.95 ± 1.84 U/mL occurred at 48 h with the pH of the medium 9.0, and an inoculum density of $2.6 \times 10^8$ cells $mL^{-1}$ at 45°C by *B. amyloliquefaciens.*

**Keywords:** Protease, biodiesel-waste derived glycerol, *Bacillus amyloliquefaciens*, *Bacillus megaterium*, *Bacillus cereus.*

## Introduction

The fossil fuel reserves are thought to become completely depleted by 2050, hence a gradual decline in petroleum production is expected in the near future. Biodiesel, a source of renewable energy has been receiving great attention because of its low cost when compared with the rising petroleum price and its impact on the environment. Biodiesel is produced by transesterification of vegetable oils or animal fat, with ethanol or methanol, catalyzed by acid or alkali. This process generates about 10 % (w/w) of glycerol as the principal by-product[2,3]. As the biodiesel production is increasing exponentially, the crude glycerol generated from the transesterification of vegetable oils has also been generated in large quantities. Thus, crude glycerol has become an abundant carbon source for bioprocess industries[4]. The usage of this crude glycerol is a big challenge, as it cannot be used for food and cosmetic uses, which are the major utilities of glycerol. Clearly, the development of processes to convert crude glycerol into value added products is a vital need[1].

Proteases have attracted a great deal of attention due to their massive industrial applications, particularly in the fertilizer, detergent, leather, pharmaceutical industries, and in the treatment of industrial waste. This enzyme accounts for about 65% of the total sales of industrial enzymes in the world market[5]. Therefore a study was performed on the production of proteases from three *Bacillus* sp. using medium containing biodiesel waste derived semipurified glycerol as the carbon source. The three *Bacillus* sp. were known to be high yielding strains of protease, with different carbon sources. In the current literature, there was no work done on using biodiesel waste derived crude glycerol as a carbon source for protease production. Hence the present investigation was aimed at optimizing the process conditions and medium components for the production of protease by the three *Bacillus* sp.

## Materials and Methods

### Microorganisms and Culture Conditions

The bacterial strains *Bacillus amyloliquefaciens* (MTCC 610)*, Bacillus megaterium* (MTCC 2949) were obtained from Microbial Type Culture Condition, IMTECH, Chandigarh, and *Bacillus cereus* BHE5400, isolated in the Department of Biotechnology, Sri Venkateswara College of Engineering, Sriperumbudur, TamilNadu, India was maintained in nutrient agar medium. The microbial strains were inoculated onto the nutrient agar plate and kept for incubation at 37°C for 24 h in an incubator (Model No: 21650, M/S. Matri Instruments

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and Private Limited, Pondicherry, India.) to obtain individual colonies of the cultures. A single colony from the nutrient agar plate was picked up and inoculated into a sterile test tube containing 5 mL of nutrient broth. The test tubes were incubated for 24 h at 37°C in a orbital shaking incubator (Model No:CIS-24 BL, M/S. Remi Instruments limited, Mumbai, India) at 200 rpm. One mL of this culture containing 19 x $10^5$ CFU of *B. amyloliquefaciens*, 2.8 x $10^5$ CFU of *B. megaterium* and 102 x $10^5$ CFU of *B. cereus* was used as inoculum in further studies. All the chemicals were purchased from Himedia Laboratories, Mumbai, India.

#### Culturing of *Bacillus sp.* in the Production Medium

The production medium containing analytical grade glucose 1% (w/v), peptone 0.5% (w/v), potassium dihydrogen phosphate ($KH_2PO_4$) 0.1% (w/v), sodium chloride (NaCl) 0.1% (w/v) and magnesium sulphate heptahydrate ($MgSO_4.7H_2O$) 0.03% (w/v) was prepared and sterilized at 121°C for 15 min[6] in an autoclave (Model No: KAUC-A2, M/S. Kemi Lab Equipment, India). In the above-mentioned production medium, the carbon source (glucose) was replaced by analytical grade glycerol 1 % (v/v) and biodiesel waste derived semipurified glycerol 1% (v/v). The resulting three different production media were compared for the protease production. One mL of bacterial inoculum *(B.amyloliquefaciens, B.megaterium and B.cereus)* was inoculated into three 250 mL Erlenmeyer flasks containing 100 mL of three different production media. These flasks were incubated at 37°C in an orbital shaker at 200 rpm.

Samples from the culture flasks were harvested every 4 h and centrifuged (Model No: R 24, M/S. Remi Instruments Limited, Mumbai, India) at 12000 x g for 10 min. Both, the cell mass and the supernatant were separated. The supernatant was assayed for protease activity and the wet cell mass was dried overnight at 105°C and dry cell weight was measured.

#### Effect of Incubation Period on Protease Production

One hundred mL of production medium was taken in 3 sets of flasks. Each set of flask contained a different carbon source namely, analytical grade glucose 1% (w/v), semipurified glycerol 1% (v/v) and analytical grade glycerol 1% (v/v). They were inoculated with1 mL of 24 h old *B. amyloliquefaciens* culture and were incubated at 37°C at 200 rpm. The above-mentioned procedures were repeated with cultures of *B. megaterium and B. cereus.* Two mL of samples were withdrawn at 4 h interval during 0-72 h of incubation. These samples were used for analyzing the protease activity and dry cell weight.

#### Effect of Semipurified Glycerol on Protease Production

One hundred mL of production medium containing peptone 0.5% (w/v), $KH_2PO_4$ 0.1% (w/v), NaCl 0.1% (w/v) and $MgSO_4.7H_2O$ 0.03% (w/v), various concentrations of semipurified glycerol *viz.*, 0.5, 1, 1.5 or 2% was added. The media was sterilized, inoculated with 1 mL of inoculum (*B. amyloliquefaciens, B. megaterium* or *B. cereus*) and incubated at 37°C for 48 h on an orbital shaker at 200 rpm. At the end of incubation time, the cell free filtrate was harvested and used in the protease assay.

#### Effect of pH on Protease Production

One hundred mL of production medium having semipurified glycerol as the carbon source was taken for this experiment. The pH of the medium was adjusted to 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, or 12.0 using 1N sodium hydroxide and 1N hydrochloric acid. The medium with different pH was inoculated with 1 mL of inoculum *(B. amyloliquefaciens, B. megaterium* or *B. cereus)* and incubated at 37°C for 48 h (at 200 rpm). At the end of incubation, the cell free filtrate was harvested and used in the protease assay.

#### Effect of Temperature on Protease Production

One hundred mL of production medium containing semipurified glycerol was sterilized and inoculated with 1 mL of bacterial inoculum (*B. amyloliquefaciens, B. megaterium* or *B. cereus*). These flasks were incubated at temperature 37, 42, 45, 48 or 51°C for 48 h. At the end of incubation time, the cell free filtrate was harvested and used in the protease assay.

#### Effect of Volume of Inoculum on Protease Production

One hundred mL of production medium containing semipurified glycerol in 250 mL Erlenmeyer flask was sterilized. Flasks containing this medium were inoculated with different volumes of inoculum *(B. amyloliquefaciens, B. megaterium* or *B. cereus)* 0.5, 1, 2, 3 or 4 % and incubated at 37°C for 48 h (at 200 rpm). At the end of incubation time, the cell free filtrate was harvested and used in the protease assay.

#### Protease Assay

Protease production was measured in terms of its proteolytic activity using caesin assay[7]. Casein was hydrolyzed into its constituent amino acids by a

protease. The phenolic hydroxyl group of the liberated tyrosine amino acid reacts with the molybdenum of the Folin - Ciocalteau (F-C) reagent in alkaline condition giving a blue colour. The absorbance of the developed colour was measured at 660 nm. Various concentrations of tyrosine were used to generate a tyrosine standard plot.

Two mL tyrosine solution of varying concentration was taken in 10 empty dry test tubes. The concentrations ranging from 0.2-2.0 µ mol/mL with an increment of 0.2 µ mol/mL were prepared from a 1.1 mM L-tyrosine stock solution. One test tube with 2 mL of distilled water (instead of tyrosine solution) was set up as the blank. Five mL of 500 mM sodium carbonate was added to the test tubes, immediately followed by the addition of 1 mL of 20% F-C reagent. They were incubated at 37°C for 30 min. The developed color was measured at an absorbance of 660 nm using a spectrophotometer (UV-VIS 118, Systronics India Limited, India). A standard plot was plotted using the absorbance obtained versus tyrosine concentration.

To 1 mL of the cell free filtrate, 5 mL of 0.65% casein solution was added, and the mixture was incubated at 37°C for 10 min. The reaction was stopped with the addition of 5 mL of 110 mM trichloro acetic acid and incubated at 37°C for 30 min. The reaction mixture was filtered through Whatman No.1 filter paper. Five mL of 500 mM sodium carbonate, immediately followed by 1mL of 20% F-C reagent was added to the filtrate and incubated at 37°C for 30 min. The developed colour was measured at an absorbance of 660 nm. One unit of protease activity is defined as the amount of enzyme required to release 1 µmole of tyrosine per min under experimental conditions.

**Production Process of Biodiesel Derived Waste Glycerol**

Waste cooking oil (SVS sunflower oil, Chennai) was collected from the college hostel kitchen, Sri Venkateswara College of Engineering, Sriperumbudur, TamilNadu India. The suspended food particles were removed by filtration with Whatman filter No 1.

The free fatty acid (FFA) content of the oil was determined by a standard titrimetric method[8]. One gram of oil was taken in an empty dry beaker and it was heated to 100°C to remove any moisture present in it. Ten mL of methanol was added to the beaker and was titrated against 0.1N sodium hydroxide. Titre value indicates the amount of sodium hydroxide required to neutralize the FFA content of the oil. The amount of sodium hydroxide required for the production of biodiesel will be the sum of the sodium hydroxide required to neutralize the FFA content of the oil (calculated for one liter of oil) and 0.5% (w/v) of oil, required as catalyst [9].

**Crude Glycerol as By-product in the Transesterification Process of the Oil**

One litre of the filtered oil was taken in the three necked round bottom flask. The first neck was fitted with a thermometer. The second was fitted with the stirrer. The third neck was covered with a cork. Sodium hydroxide mixed with methanol was added through this neck. The flask was heated to 100°C using a heating mantle to remove the moisture content of the oil and then brought to 55°C, a temperature lesser than the boiling point of methanol. This is done to avoid the evaporation of methanol when added to the oil. Three gram of NaOH dissolved in 200 mL methanol was poured into the flask and stirred at 500 rpm for 2 h at 55 ± 5°C. The contents were allowed to stand for 2 h, after which the reactant mixture separated into two layers. The brownish red colored bottom layer contained crude glycerol and the lemon yellow colored upper layer contained the esters (biodiesel). The top and the bottom layer were separated using the separating funnel[10].

The crude glycerol contained impurities such as water, methanol, salts and fatty acids. It was dark brown in color with the pH ranging between 10.5 and 11. This crude glycerol was acidified by the addition of 45N phosphoric acid to bring down the pH to 2.5. It was left undisturbed for 12 h in a separating funnel until the solution separated into two distinct phases comprising of an FFA layer on the top and a glycerol-rich layer at the bottom. The glycerol rich layer was separated and neutralized to a pH of 7 by the addition of 5N NaOH. It was then mixed with methanol in the ratio of 3:1 (v/v) by shaking for 10 min and left undisturbed for 2 h to enhance the precipitation of sodium phosphate salts. A two phase solution was obtained, the glycerol–alcohol phase on the top and the crystallized salt in the bottom.

The crude glycerol contaminated with methanol was heated to 70°C for 30 min to obtain methanol free glycerol. This glycerol was treated with activated charcoal, which acts as a decolorizing agent. Using a magnetic stirrer (2MLH, Remi Instruments Limited, Mumbai, India) 1 g of activated charcoal was mixed with 50 mL of crude glycerol obtained from the above

process, at 200 rpm for 30 min. Then, the solution was filtered using Whatman filter No 1 to remove the activated charcoal leaving behind the clear semipurified glycerol.

#### Glycerol Estimation

The purity of this semipurified glycerol was determined using dichrometry method[11]. Ten mL of the analytical grade glycerol was taken in a conical flask. Thirty mL of 0.1N potassium dichromate and 20 mL of dilute sulfuric acid were added to it. It was placed in a boiling water bath for 1 h and cooled. Ten mL of 10% potassium iodide was added to the above mixture. The liberated iodine was titrated against 0.1 N sodium thiosulphate solution, using starch as indicator. The end point was the appearance of emerald green color. From the titer value, the amount of glycerol present in the given sample was calculated. The above procedure was repeated with semipurified glycerol and the titer values obtained were used to estimate the purity of semipurified glycerol.

#### Statistical Analysis

All experiments were conducted in duplicates and the analyses were performed in triplicates. Students T test was performed to confirm the significant difference between the various treatments. A p-value less than 0.05 was considered as significant.

## Results and Discussion

#### Free Fatty Acid

The free faty acid content of every batch of waste cooking oil was determined. It was found that on an average 1g of oil consumed 1.6 mg of NaOH. Therefore, 1kg of oil requires 1.6 g NaOH to neutralize the FFA content. Moreover, 0.5% of catalyst i.e 5g NaOH/kg of oil was required for the complete transesterification of the oil. Methanol in the presence of sodium hydroxide (catalyst) reacts with oil to give biodiesel and crude glycerol. Sodium hydroxide does not dissolve readily and completely when added to oil at 70°C, hence it is dissolved in methanol and then added to the oil.

Oil to methanol ratio of 5:1was found to yield maximum biodiesel. A ratio of 10:1 yielded very less conversion of oil to biodiesel and when the ratio was 3:1, the amount of methanol was in excess and there was no increase in conversion levels. During the biodiesel production, the amount of crude glycerol generated was found to be 118 ± 15 mL.

#### Refining the Crude Glycerol

The crude glycerol was acidified to yield different pH such as 1, 2.5, 4, 5, 6 and 7 using 45N phosphoric acid. The crude glycerol phase separated into two or three distinct layers comprising of an FFA layer on the top, a glycerol-rich layer in the middle (for pH 4, 5, 6, and 7) and an inorganic salt layer at the bottom. This was also observed by Kongjao *et al.* (2010)[12]. When the pH was reduced below 4 the salts dissolved back into the glycerol-rich layer, due to the solubility of salts in a highly acidic medium.

The glycerol-rich layer obtained from the above acidification stage was then subjected to solvent extraction process using methanol as a solvent in the ratio 3:1. The mixture was left undisturbed overnight. Sodium phosphate salts have the least solubility in methanol, hence aids the separation of the salts effectively. The top layer containing glycerol and methanol was decanted and the crystallized salts were clearly observed at the bottom of the beaker. Similar observations were made by Manosak *et al.* (2011)[13] and Kongjao *et al.* (2010)[12].

By heating the top layer, the excess methanol and the moisture present was evaporated leaving the crude glycerol in light brown color. This color may be due to the presence of saturated fatty acids and some ash since waste cooking oil was used to produce biodiesel. This crude glycerol yielded a colorless semipurified glycerol when treated with activated charcoal. Activated charcoal removed the impurities that gave a brown color to the crude glycerol. The semipurified glycerol obtained after sequential refining was clear and highly viscous compared to crude glycerol obtained as a byproduct from biodiesel production. This semipurified glycerol was stored in an air tight container for further use. The glycerol content in the semipurified glycerol was estimated by dichrometry method using the procedure detailed in the methodology. Advanced techniques to estimate the glycerol content will be used in further experiments.

The range of glycerol purity in the semipurified glycerol samples ranged from 81.36% to 84.75 % (82.94 ± 1.7 %). Hajek and Skopal (2010)[14] reported 86% purity with the crude glycerol they procured from Lachner, Czech Republic. Manosak *et al.* (2011)[13] showed a purity of 96.2 % with crude glycerol obtained from a local biodiesel production plant in Thailand. Kongjao *et al.* (2010)[12] reported a purity of 93.3% with the crude glycerol obtained from transesterification of

waste used oil. The purity of the glycerol in this study (88.2%) was less when compared with that reported by other researchers. This may be due to the variation in the quality of the used waste cooking oil obtained in batches (different days of the week) from the hostel to produce biodiesel. As stated by other researchers, increased water content in the glycerol phase also would result in less purity of the final glycerol content.

Though NaOH is reported to be an effective catalyst in the transesterification process compared to KOH, the salt obtained finally is $NaH_2PO_4$ which has a low value when compared to $KH_2PO_4$ that could be used as a fertilizer. Hence it is preferred to use KOH as a catalyst during the transesterification reaction.

**Effect of Culturing Period on Cell Growth**

A study was performed to understand the influence of incubation time on the growth of the microorganisms (*B. amyloliquefaciens, B. megaterium* and *B. cereus*) using the media with three different carbon sources. The exponential growth phase of these microbes started at about 8 h after inoculation and reached the maximum at 24 h. Later stationary phase started and lasted till 48 h of incubation. The dry cell weight produced by the microorganisms with three different carbon sources namely analytical grade glycerol, semi-purified glycerol and analytical grade glucose is given in Table1.

Using analytical grade glycerol as the carbon source a maximum dry cell weight of 6.1 ± 0.15 mg/mL was obtained at 40 h of incubation by *B. amyloliquefaciens.* But in the case of *B. megaterium* the maximum dry cell weight was 5.3 ± 0.14 mg/mL obtained at 44 h. After 40 h of incubation, the maximum amount of dry cell weight obtained by *B. cereus* was 5 ± 0.32 mg/mL. When the amount of dry cell weight produced by *B. amyloliquefaciens* was compared with *B. megaterium* using a student's T test, there was a significant increase in dry cell weight by *B. amyloliquefaciens*. When the amount of dry cell weight produced by *B. megaterium* and compared with *B. cereus*, there was no significant difference between their weights. ($p > 0.05$). Therefore, *B. amyloliquefaciens* was found to produce a significantly high amount of dry cell weight when compared with *B. cereus* and *B. megaterium.*

Table 1, shows the dry cell weight produced by *B. amyloliquefaciens*, *B. megaterium* and *B. cereus* when cultured in the media containing semipurified glycerol as the carbon source. *B. amyloliquefaciens*, *B. megaterium* and *B. cereus* produced a dry cell weight of 4.9 ± 0.13 mg/mL, 4 ± 0.06 mg/mL and 4±0.03 mg/mL respectively at 40 h of incubation. When the production of cell mass was compared among the three organisms, *B. amyloliquefaciens* has shown a significantly higher cell mass production when compared with *B. megaterium or B. cereus* ($p < 0.05$), whereas cell mass production by *B. megaterium* and *B. cereus* were similar.

Table: 1, shows the dry cell weight produced by the three organisms in media containing analytical grade glucose as the carbon source. In this media, a dry cell weight of 6.4 ± 0.05 mg/mL was obtained at 40 h of incubation by *B. amyloliquefaciens.* But *B. megaterium* produced 6 ± 0.05 mg/mL of dry cell mass even after 44 h. *B. cereus* produced 6.2 ± 0.14 mg/mL dry cell weight at 40 h of incubation. When the difference in production of dry cell weight was compared among the three species mentioned above, it was found that the amount of cell mass produced by *B. amyloliquefaciens* was significantly higher ($p < 0.05$) than that of *B. megaterium* and that of *B. cereus*.

*B. amyloliquefaciens* shows the highest cell growth with all the three carbon sources. It is also seen from the above table that the dry cell weights were significantly less in the semipurified glycerol medium when compared to that of analytical grade glucose and analytical grade glycerol, irrespective of the

Table — 1 Effect of culturing period on cell growth with three different carbon sources

| Carbon Source Organism | Analytical Grade Glucose | | Analytical Grade Glycerol | | Semipurified Glycerol | |
|---|---|---|---|---|---|---|
| | Optimum Incubation Time (h) | Dry Cell Weight (mg/ml) | Optimum Incubation Time (h) | Dry Cell Weight (mg/ml) | Optimum Incubation Time (h) | Dry Cell Weight (mg/ml) |
| *B.amyloliquefaciens* | 40 | 6.4 ± 0.05 | 40 | 6.1 ± 0.15 | 40 | 4.9 ± 0.13 |
| *B.megaterium* | 44 | 6 ±0 .05 | 44 | 5.3 ± 0.14 | 44 | 4 ± 0.06 |
| *B.cereus* | 40 | 6.2 ± 0.14 | 40 | 5 ± 0.32 | 40 | 4 ± 0.03 |

organisms. This effect could be attributed to the presence of trace quantity of impurities including salts in the semipurified glycerol. However, Gupta *et al.* (2007)[15] has reported that glycerol was a better carbon source for the production of protease when compared with sucrose maltose and fructose.

**Effect of Culturing Period on Protease Activity**

The increase in the culturing period of these microorganisms showed an increase on the production of protease. *Bacillus* sp. are known to produce the maximum amount of proteases in the stationary phase or post exponential phase of their growth[16, 17]. This was verified in our study.

Initial optimization of incubation period was studied for 0-72 h for all the three species. This study showed 48 h of incubation for *B. amyloliquefaciens* produced the highest concentration of protease. Therefore, this 48 h incubation time was used in the rest of the experiment with *B. amyloliquefaciens*. Similarly, 44 h and 40 h of incubation were found optimum for *B. megaterium* and *B. cereus* respectively, therefore was used in the rest of the experiments. As shown in the Table 2**,** a maximum protease activity of 308.08 ± 5.91 U/mL was observed for *B. amyloliquefaciens* at 48h with analytical grade glycerol. The maximum protease activity (289.3 ± 13.84 U/mL) was observed with *B. megaterium* at 44 h. A maximum activity of 270.14 ± 8.94 U/mL was observed with *B. cereus* at 40 h. The protease activity by *B. amyloliquefaciens* was statistically higher than that by *B. megaterium* ($p < 0.05$). Between *B. megaterium* and *B. cereus,* the former was a significant producer ($p < 0.05$) of protease.

As seen in the Table 2, with semipurified glycerol as the carbon source *B. amyloliquefaciens*, *B. megaterium* and *B. cereus* produced a protease activity of 294.98 ± 21.01 U/mL, 229.9 ± 11.0 U/mL and 203.23 ± 11.0 U/mL respectively at 48 h, 44 h, and 40 h. *B. amyloliquefaciens* was producing a significantly higher protease activity compared to *B. megaterium* ($p < 0.05$). *B. cereus* was producing a significantly higher protease activity compared to *B. megaterium* ($p < 0.05$).

As shown in Table 2, with analytical grade glucose as the carbon source, *B. amyloliquefaciens*, *B. megaterium* and *B. cereus* produced a protease activity of 343.57 ± 20.97 U/mL, 334.77 ± 11.56 U/mL and 300.67 ± 15.31 U/mL, respectively. There was no significant difference in protease activity between *B. amyloliquefaciens* and *B. megaterium* ($p > 0.05$). But with *B. cereus* the protease production was significantly lower ($p > 0.05$). With 1% glycerol and 1% glucose, *B. cereus* showed the same protease activity, as reported by Uyar *et al*. (2011)[18]. *B. amyloliquefaciens* and *B. megaterium* showed a protease activity of 531.14 U/mL and 512.24 U/mL respectively with glucose as the carbon source. The amount of protease production, reported by Boominadhan *et al.* (2009)[19] is higher than the maximum activity observed in the present work. This may be due to the reason that *B. megaterium* preferred lactose as the best carbon source as confirmed by Rajkumar *et al.* (2010)[20].

Among all the three species compared, *B. amyloliquefaciens* gave the highest protease activity with all the three carbon sources. It could be observed that semipurified glycerol yielded the lowest protease activity in all the three species, compared to the other carbon sources. Analytical grade glucose was the carbon source that yielded a higher protease activity. This could be attributed to the fact that impurities present in the semipurified glycerol affected both the growth of the organism as well as protease production. Further purification of the semipurified glycerol might lead to increased protease activity.

Table — 2 Effect of culturing period on protease activity with three different carbon sources

| Carbon Source | Analytical Grade Glucose | | Analytical Grade Glycerol | | Semipurified Glycerol | |
|---|---|---|---|---|---|---|
| Organism | Optimum Incubation Time (h) | Protease Activity (U/Ml) | Optimum Incubation Time (h) | Protease Activity (U/ml) | Optimum Incubation Time (h) | Protease Activity (U/ml) |
| *B.amyloliquefaciens* | 48 | 343.57±20.97 | 48 | 308.08 ± 5.91 | 48 | 294.98 ± 21.01 |
| *B.megaterium* | 44 | 334.77 ± 11.56 | 44 | 289.3 ± 13.84 | 44 | 229.9 ± 11.0 |
| *B.cereus* | 40 | 300.67 ± 15.31 | 40 | 270.14±8.94 | 40 | 203.23 ±11.0 |

The protease activity did not increase significantly after the optimum culturing period, even though there was a constant increase in the dry cell weight, irrespective of the species and carbon source. A similar observation was made by Boominadhan *et al.* (2009)[19]. An optimum incubation period of 48 h was observed best for protease activity, after which there was no significant increase.

**Effect of Semi - purified Concentration on Protease Activity**

The effect of four different concentrations of semipurified glycerol on the production of protease by the three species, namely *B. amyloliquefaciens*, *B. megaterium* and *B. cereus* was studied, to understand the organism that yields the highest protease activity.

Figure 1 shows protease production by three organisms at three different semipurified glycerol concentrations. semipurified glycerol of 1% concentration was found to be optimum for protease production by *B. amyloliquefaciens* (294.8 ± 12.95 U/mL), *B. megaterium* (229.9 ± 11.26 U/mL) and *B. cereus* (209.4 ± 8.59 U/mL). Semipurified glycerol of 0.5 % concentration yielded significantly less protease compared to 1%, by all the three organisms ($p < 0.05$). Among the three *Bacillus* species, *B. amyloliquefaciens* was found to produce the highest protease activity with 1 % glycerol concentration. The study indicates that concentration of semipurified glycerol more than 1 % could inhibit the protease production, by all the three species. Qadar *et al.* (2009)[21] observed a similar result with *Bacillus subtilis,* where 1% glucose concentration yielded a maximum protease activity of 128 U/mL. Moreover, Gupta *et al.* (2007)[15] reported that 0.7% analytical grade glycerol produced maximum protease activity by *Pseudomonas aeruginosa* PseA. Siddique *et al.* (2001)[22] showed that 1% glycerol was optimum for producing the maximum protease activity by *Streptomyces thermoviolaceus.*

**Effect of pH on Protease Activity**

The pH of the medium is a very crucial parameter, as it strongly affects many enzymatic processes and transport of compounds across the cell membrane[4]. In the present study among the different pH levels were tested, pH 8 was optimum for *B. amyloliquefaciens* and *B. megaterium,* and pH 9 was optimum for *B. cereus*, as shown in the Figure 2.

A maximum protease activity of 343.3 ± 14.41 U/mL was observed by *B. megaterium*. This was significantly higher than the protease production by the other two species ($p < 0.05$). In case of *B. amyloliquefaciens* the protease production at pH 9 was significantly higher than pH 8 ($p < 0.05$), but the production at pH 9 and 10 did not vary significantly ($p > 0.05$). The protease production by *B. megaterium* (343.3 ± 14.41 U/mL) was higher than that of *B. amyloliquefaciens* (300.67 ± 17.28 U/mL) and at the same pH.

Boominadhan *et al.* (2009)[19] also reported that a pH of 8 was optimum for the protease production by all their *Bacillus* isolates. Moreover, Praveen *et al.* (2008)[23] reported that a pH of 8 was optimum for protease production for their *Bacillus* isolates. In the present study, protease activity did not increase further after the optimum pH value, instead started decreasing for all the three organisms. This is because the further increase in pH was not favorable for the growth and metabolic activity of all the organisms.

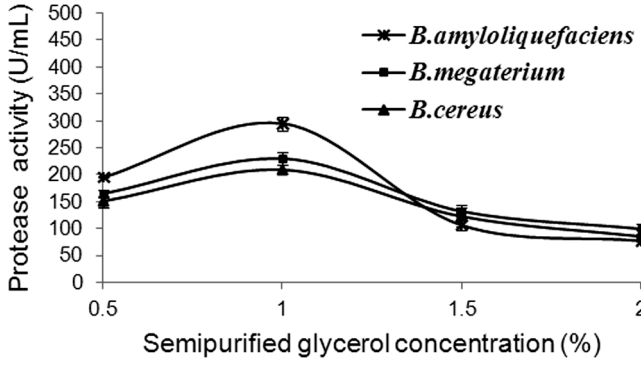


Fig.1 — Effect of semipurified glycerol concentration on protease activity

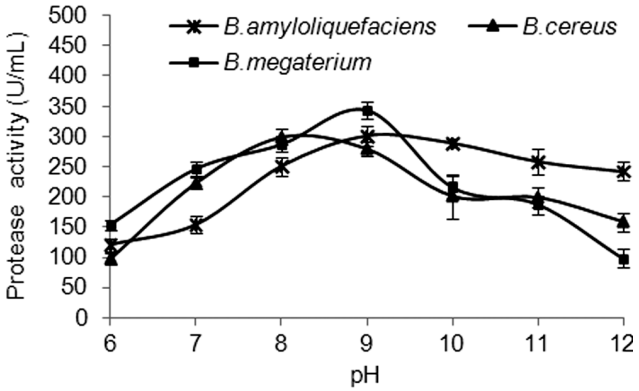


Fig.2 — Effect of pH on protease activity

**Effect of Temperature on Protease Activity**

The influence of incubation temperature on protease production by *Bacillus* species was studied by different workers[19,24]. The optimum temperature for the growth of the organism and its protease production varied from one organism to the other. Therefore, this study used different incubation temperatures for protease production. As shown in Figure 3, at an optimum temperature of 45ºC *B. amyloliquefaciens* yielded the maximum protease activity of 543.95 ± 6.34 U/mL. The protease activity at the other temperatures had a significantly lesser production ($p < 0.05$). *B. megaterium* produced a maximum of 450.9 ± 11.25 U/mL at a temperature of 40ºC, which was statistically significant when compared to the protease production at 37ºC and 43ºC ($p < 0.05$). The temperature requirement for *B. cereus* was the highest among all the three species. At 50ºC, the maximum protease activity was 469.43 ± 9.08 U/mL. This protease activity was higher when compared to that produced at 48ºC ($P < 0.05$).

In the present study, the optimum temperature for protease production with semipurified glycerol as the carbon source was found to be 45°C for *B. amyloliquefaciens*, 40°C for *B. megaterium* and 50°C for *B. cereus* as shown in Figure 3. Sen and Satyanarayana (1981)[25], showed that the optimum temperature for protease production by thermophilic *B. licheniformis* S-40 was 45°C and for *B. subtilis* 50°C. The protease production decreased above the optimum temperature in all the three organisms. Cell growth was inhibited above the optimum temperature due to the inactivation of the enzyme activity in glycolysis and the Krebs' cycle, and then consequently decreased the carbon metabolism [26].

**Effect of Volume of Inoculum on Protease Production**

As shown in Figure 4, irrespective of the *Bacillus* sp., the maximum protease production was obtained with 1% inoculum with crude glycerol as carbon source. *B. amyloliquefaciens* showed a maximum protease production of 543.95 ± 10.26 U/mL was obtained with 1% inoculum. This production is significantly higher than that produced with 0.5% and 2% inoculum size ($p < 0.05$). The same trend was observed with both *B. megaterium* and *B. cereus.*

Higher inoculum sizes resulted in the decreased protease production, which could be due the nutrient depletion in the medium as reported by Rahman *et al.* (2005)[27]. These results showed close resemblance

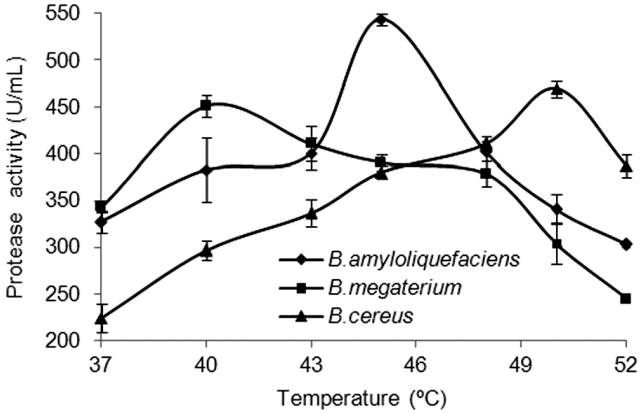


Fig.3 — Effect of temperature on protease activity

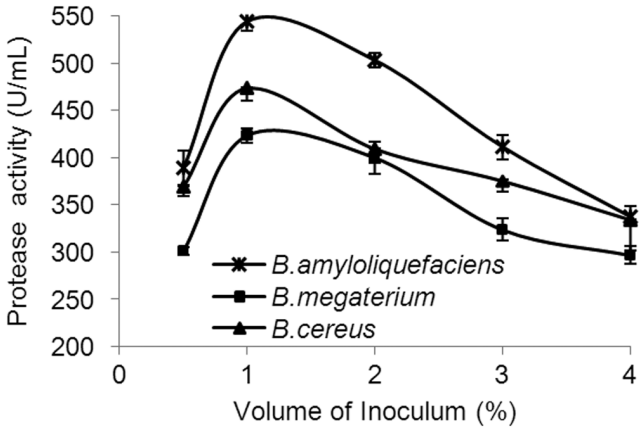


Fig. 4 — Effect of volume of inoculum on protease activity

with the alkaline protease production by thermophilic *B. licheniformis* using glycerol as carbon source[28]. Moreover, Nilegoankar *et al.* (2007)[29] also reported that maximum protease production was with 1% inoculum of *B. cereus* MCM B-326.

## Conclusion

Crude glycerol from the biodiesel production was purified to semipurified glycerol that contains 82.94 ± 1.7% glycerol, using a small variation in the established process. The results of this study show that this semipurified glycerol could be used as a sole carbon source for microbial fermentation. Successful application of this work could have a significant impact on the biodiesel industry as it helps to solve the problem of crude glycerol disposal, while simultaneously producing a high value product. Initial glycerol concentration, incubation period, pH and temperature, the volume of inoculum had profound effects on protease production from *Bacillus* sp. Among the three *Bacillus* sp. studied, *B. amyloliquefaciens* was found to the best producer of

protease, using biodiesel waste derived semipurified glycerol as carbon source. The protease also exhibited high activity even under alkaline pH, at broad temperature range utilizing the novel substrate, semipurified glycerol obtained from biodiesel production, and thus making the enzyme potentially useful in various industries. The protease production reported in this present work is significantly higher than that reported by many other researchers. Hence this conversion of biodiesel derived semipurified glycerol in to protease will be of commercial importance. This semipurified glycerol obtained from biodiesel production proves as a promising carbon source, which could be studied for its conversion into other potential products.

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