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Study of nutrient media components and cultivation conditions of *Bacillus licheniformis* BCC-02-50 for protease production using molasses as energy source

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Abstract

An efficient thermotolerant strain *Bacillus licheniformis* was used in this study for optimizing different variables (time period, carbon and nitrogen sources, temperature, pH, age and size of inoculum) for protease production using one variable at one time. All the variables were found significantly influencing protease production individually and in interaction with other variables. The study predicted protease production of 2356 U/mL after 36 h of incubation and further enzyme concentration increased up to 4387 U/mL when molasses (20 g/L) and yeast extract (10 g/L) were used as sole carbon and nitrogen source. More increase in extracellular protease secretion was noted in alkaline pH 10.0 (6463 U/mL) and 7528 U/mL at temperature 48 °C. Results suggest practical approach for protease production for local detergent preparations and leather industries.

Key Words: Protease, molasses, *Bacillus licheniformis*, thermophilic, alkaliphilic.

Introduction

Utilization of enzymes in industrial sector and especially proteases in detergents, leather and food industries has greatly increased its demands (Ibrahim et al., 2015). Global markets for industrial enzymes was $3.3 billion in 2010 and $4.4 billion in 2015 and continuously increasing. In particular, global financial crises can push optimistic calculations in the opposite direction or, at best, sideways. Due to the looming climate catastrophe, the search for green industrial processes is more urgent and popular, and the search for robust enzymes is a key feature of this urgent effort (Raval et al., 2014).

Protease catalyzes the breakdown of proteins to simpler peptides and finally to amino acids. Proteases are most important industrial enzymes that accounts 65% of total industrial enzyme market (Ibrahim et al., 2015). Alkaline proteases are most demanded due to their specificity, stability toward pH, salt tolerance, thermostability

and stability in presence of organic solvents, metal ions and surfactants. Hence alkaline proteases producing organisms have gained more attention of the scientific community.

Proteases are secreted in all forms of life including plants, animals, microorganisms (fungi and bacteria) (Salihi et al., 2017). Proteases obtained from bacterial strains represent great diversity for activity, thermostability, stability in wide range of pH , salt and organic solvent tolerance (Asker et al., 2013). Several *Bacillus* species have been reported for protease production, for example, *Bacillus cereus*, *Bacillus sterothermophilus*, *Bacillus mojavensis*, *Bacillus megaterium* and *Bacillus subtilis* (Mohsen et al., 2013). Fungal strains also exploited for protease production such as, *Aspergillus flavus*, *Aspergillus niger* (Devi et al., 2008*)*, *Aspergillus clavatus* (Hajji et al., 2008), *Aspergillus oryzae* (Srinubabu et al., 2007) , *A. terreus* (Sethi et al., 2013) *Aspergillus awamori* (Negi and Banerjee, 2010) and *Aspergillus fischeri* (Saravanakumar et al., 2010). Many studies have evaluated the influence of nutritional components such as carbon and nitrogen source on microbial protease production (Neves et al., 2006; Santiago and Motta, 2008). Furthermore, there are several studies in which agro-industrial by-products (soybean meal, rice bran, and wheat bran) were used as energy source for biosynthesis of protease (Mukhtar, 2009; Radha et al., 2017). In addition, physico-chemical parameters including temperature and pH have also enhanced protease production by microorganisms (Chaud et al., 2016).

In this study, extracellular protease production from *Bacillus licheniformis* in submerged fermentation using molasses as cost-effective energy source was evaluated. The strain is thermophilic and alkalophilic in nature that could allow open nonsterilized fermentation at laboratory and pilot scale production. In our previous studies (Qureshi et al., 2016), we performed open non-sterilized solid substrate fermentation to produce amylase and protease. Similar results can be obtained from this strain. Thus, open nonsterilized fermentation will simplify enzyme production technology and save sterilization cost and energy.

Materials and Methods

Microorganism

More than 50 bacterial strains were isolated from soil and screened for protease production on casein agar plates (Casein 4 g, peptone 4 gram, Agar 4 g dissolved in 200 mL distilled water). Medium was sterilized and poured in petri plates. Then, each culture was spread on the agar plates and allowed to grow for 48 h then (1% w/v) tannic acid solution was flooded on the plates and clear zone formation was observed. Based on larger zone, strains were selected for fermentation experiments. Best strain was identified as *Bacillus licheniformis* according to morphological and biochemical analysis and subsequently used in all fermentation experiments.

Culture conditions

First of all, strain was taken from the stock and grown in synthetic medium containing 20 g/L of glucose, 5 g/L of yeast extract, 5 g/L of peptone, and 5 g/L of NaCl flasks were incubated for 24 h at 37 °C (180 rpm). In next step, a volume of 5.0 % v/v culture was transferred into another synthetic medium that was named as seeds culture medium reported elsewhere (Simair et al., 2017) flasks were incubated in shaking conditions at 37 °C for 24 h. 1.0 % v/v inoculum from seeds culture was inoculated into final enzyme production medium, composition of fermentation medium was same as reported in (Simair et al., 2017). Flasks were incubated for 120 h at 37 °C and 180 rpm agitation speed. Culture broth obtained after centrifugation was tested for protease activity.

Effect of carbon sources

The effect of carbon source on growth and protease production was determined by replacing glucose with molasses, date syrup, galactose, fructose, maltose, starch, lactose,

and sucrose with concentration of 20 g/L in mineral medium. The inoculated flasks were incubated at 37 °C for 36 h.

Effect of Nitrogen sources

Different nitrogen sources were supplemented in fermentation medium replacing 10 g/L of peptone with yeast extract, urea, casein, meat extract, tryptone, ammonium chloride, ammonium nitrate, and ammonium sulphate. The inoculated flasks were incubated at 37 °C for 36 h.

Effect of temperature and pH

Influence of temperature on bacterial growth and fermentability was investigated by culturing microorganism at various temperatures (30–60 °C). Whereas, effect of pH on protease production was observed by changing initial pH from 5 to 12, all fermentation experiments lasted for 36 h.

Size and age of inoculum

To investigate the effect of inoculum size (0.5 to 10 % v/v) on cell growth and protease activity, 50 mL of fermentation medium was inoculated with 24 h old mature seeds culture with different inoculum sizes. Effect of age of inoculum ranging from 0 to 48 h was also checked. The inoculated flasks were incubated for 36 h.

Protease assay

Protease activity was checked according to method reported by Penner and Aston [1967], detailed method adopted from (Qureshi et al., 2011). One unit of protease activity was defined as the amount of protease required to catalyze the liberation of 1 µg of tyrosine under the assay conditions.

Result and discussion

Protease is one of commercial enzymes that could be used in food, pharmaceutical, detergent, textile industries, leather and other industries (Ibrahim et al., 2015). Proteases could be produced from recombinant strains or produced from locally isolated strains for its application in local detergents formulation. In our previous study, we have evaluated capability of thermophilic strain for protease production under nonsterilized solid state fermentation conditions. Protease of Bacillus sp BBXS-02 successfully removed the blood stains from cloth pieces (Qureshi et al., 2016) that suggested suitability of enzyme for local detergent formulations.

This research work deals with the optimization of fermentation conditions for protease production from thermophilic bacterial strain Bacillus licheniformis. Previous studies have reported that optimization of culture conditions enhance microbial performance in terms of growth and production rate (Qureshi et al., 2011; Qureshi et al., 2016; Simair et al., 2017). Another important reason of research was to evaluate cost-effective carbon source (molasses) for protease production from *Bacillus licheniformis.*

Bacterial growth and protease concentration increased with fermentation time upto 36 h then growth and enzyme titer gradually decreased due to elongated incubation period, results are shown in Figure 1. Protease concentration reduced might be due to the reduction in nutrients, accumulation of waste product, cell death, and catabolite repression (Simair et al., 2017). The effects of different carbon sources (glucose, molasses, date syrup, galactose, fructose, maltose, starch, lactose, and sucrose) were investigated on protease production (Figure 2). Maximal cell growth and protease titer were achieved when molasses (20 g/L) was used as energy source as compared to other carbon sources. In present study, molasses was found best carbon source for microbial growth and protease fermentation. This occurred probably due to the presence of growth promoters and other nutrients in molasses (Simair et al., 2017) . The obtained results are in agreement with previous studies where some carbon sources (glucose and maltose) inhibited protease production, due to catabolite repression of protein biosynthesis (Deng et al., 2010; Kanekar et al., 2002; Pathak and Deshmukh, 2012).

Various organic and inorganic compounds, peptone, yeast extract, urea, casein, meat extract, tryptone, ammonium chloride, ammonium nitrate, and ammonium sulphate, were tested as a nitrogen source. Results are shown in Figure 3 expose that addition of 10 g/L yeast extract boosted the growth of *Bacillus* sp. and protease yield. All tested organic nitrogen sources supported bacterial growth, as well as protease yield but higher protease titer of 4387 U/mL was obtained from yeast extract as compared to other nitrogen sources. This result was in agreement with that reported for marine Bacillus sp. MIG (Sanchez-Porro et al., 2003), alkaliphilic *Bacillus pumilus* MCAS8 (Jayakumar et al., 2012), alkaliphilic *Bacillus licheniformis* KBDL4 (Deng et al., 2010), and *Bacillus cluasii* (Lakshmi et al., 2014). These studies reported maximum protease titer when yeast extract was used as compared inorganic nitrogen sources. In other studies various organic nitrogen sources best supported microbial growth and protease production, for example, skim milk(Gouda, 2006), peptone (Oskouie et al., 2008), casamino acids (Jain et al., 2012), beef extract (Kumar et al., 2014).

Microbial enzymes production and extracellular secretion depends on medium pH probably due to effect of pH on enzymatic reaction and stability. Figure 4 reveals the effect of initial pH (5.0 to 12.0) on protease synthesis by *Bacillus* sp. in minimal medium containing 20 g/L of molasses and 10 g/L of yeast extract incubated at 37 °C for 36 h in shaking conditions. Microbial growth and protease production were noted highest at pH 10, which indicates alkaliphilic nature of strain (Horikoshi, 1999; Kanekar et al., 2002; Patel et al., 2005; Pathak and Deshmukh, 2012).

Effect of fermentation temperature (30-60 °C) was investigated on protease production from *Bacillus* sp.in synthetic medium containing molasses and yeast extract as carbon and nitrogen source, initial pH was adjusted to 10.0 and incubated for 36 h. Enzyme concentration increased with incubation temperature and maximum protease yield (7528 U/mL) was noted at 48 ° C, results are depicted in Figure 5.

Effect of inoculum size (0.5 to 10% v/v) on cell growth and extracellular protease secretion are shown in Figure 6. Highest protease concentration was achieved when 2 (% v/v) of 24 h old seeds culture was inoculated into the fermentation medium and growth of *Bacillus* was observed at 10 (% v/v). Whereas age of inoculum was also checked, the growth and production of protease was noted highest at 24 h old seeds culture, results are shown in Figure 7. Optimization of age and size of inoculum is very important variable because it effects on rate of production and cost of final product. This study suggested that protease production from *Bacillus* sp. is growth-dependent. The size and age of inoculum play a significant role in fermentation (Simair et al., 2017).

Conclusion

In this study, a new promising thermotolerant alkaline protease producer, *Bacillus licheniformis* strain was used. Effect of variables, i.e. time, carbon and nitrogen sources, temperature, pH, size and age of inoculum for optimizing their levels in the medium and effect of their mutual interaction on thermotolerant alkaline protease production was checked. The protease production in the optimized media was 7528 U/ml. Based on the data of the present experiment, it is suggested that significant improvement in the production of alkaline protease by *Bacillus licheniformis* strain was accomplished using cheap carbon source. The results revealed that strain *Bacillus licheniformis* showed a number of properties (pH optimum 10 and optimum temperature 48 °C) that are highly valued for application of the protease enzyme from this strain in the industrial processes.

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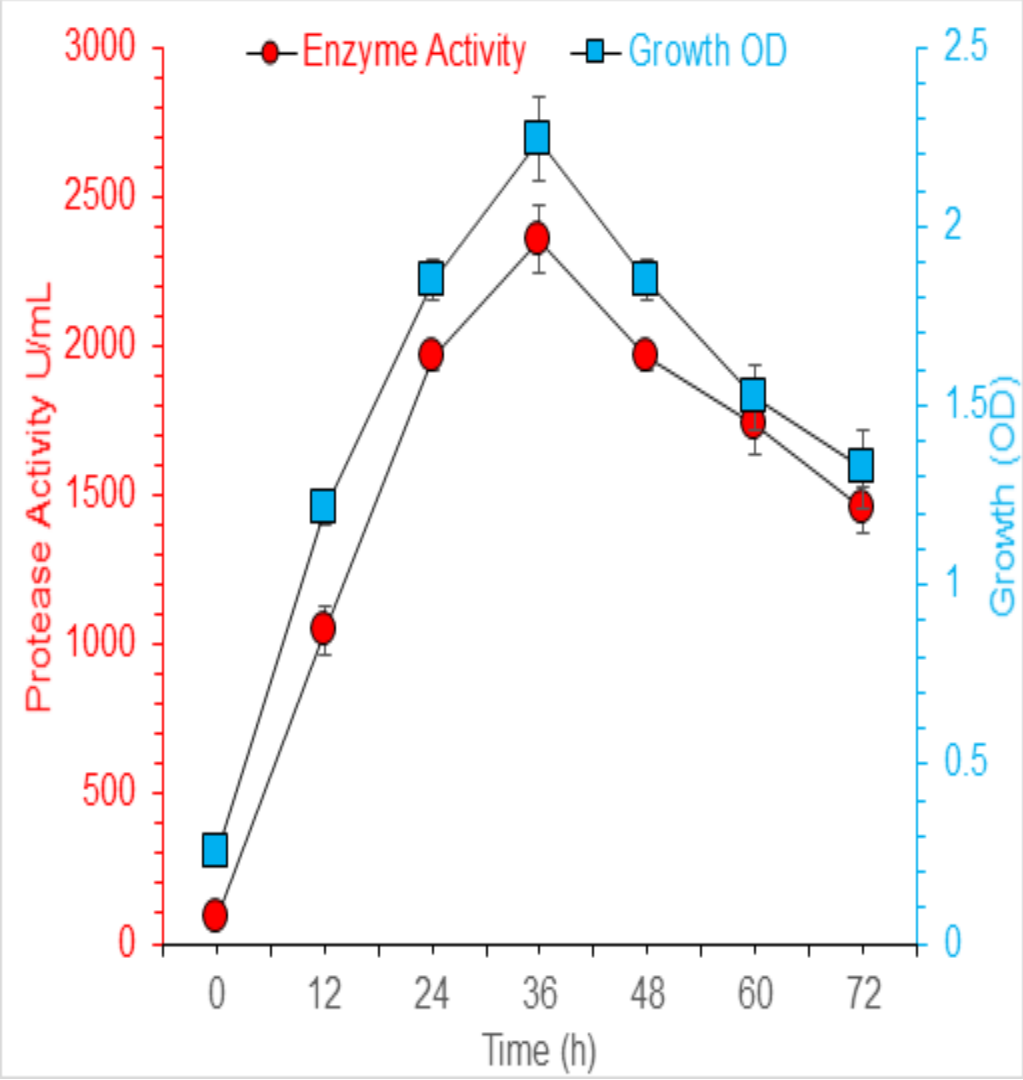

Figure 1. Batch profile of protease production from *Bacillus licheniformis* BCC-02-50 in synthetic medium containing glucose 20 g/L, yeast extract 10 g/L, $MgSO_4 \cdot 7H_2O$ 1.0 g/L, KH_2PO_4 2 g/L and incubated in shaking incubator at 37 °C with initial pH 7.0. The experiments were performed in triplicate and data presented in figures are average of three parallel experiments. Error bars are shown for standard deviation.

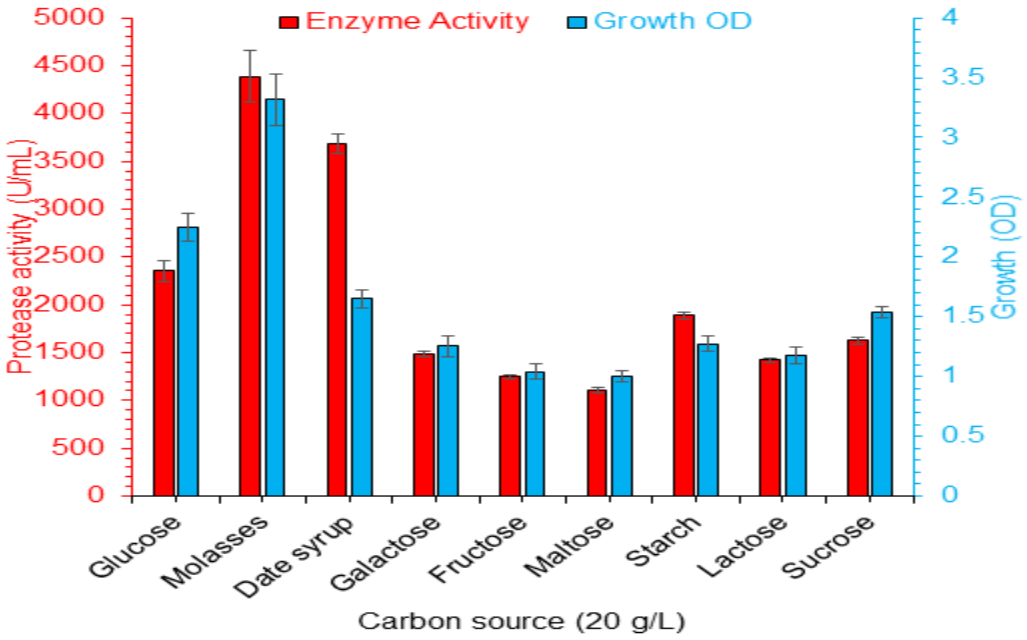

Figure 2. Effect of the carbon source (20 g/L initial concentration) on protease production at 37 °C, initial pH of 7.0 for 36 h. The experiments were performed in triplicate and data presented in figures are average of three parallel experiments. Error bars are shown for standard deviation.

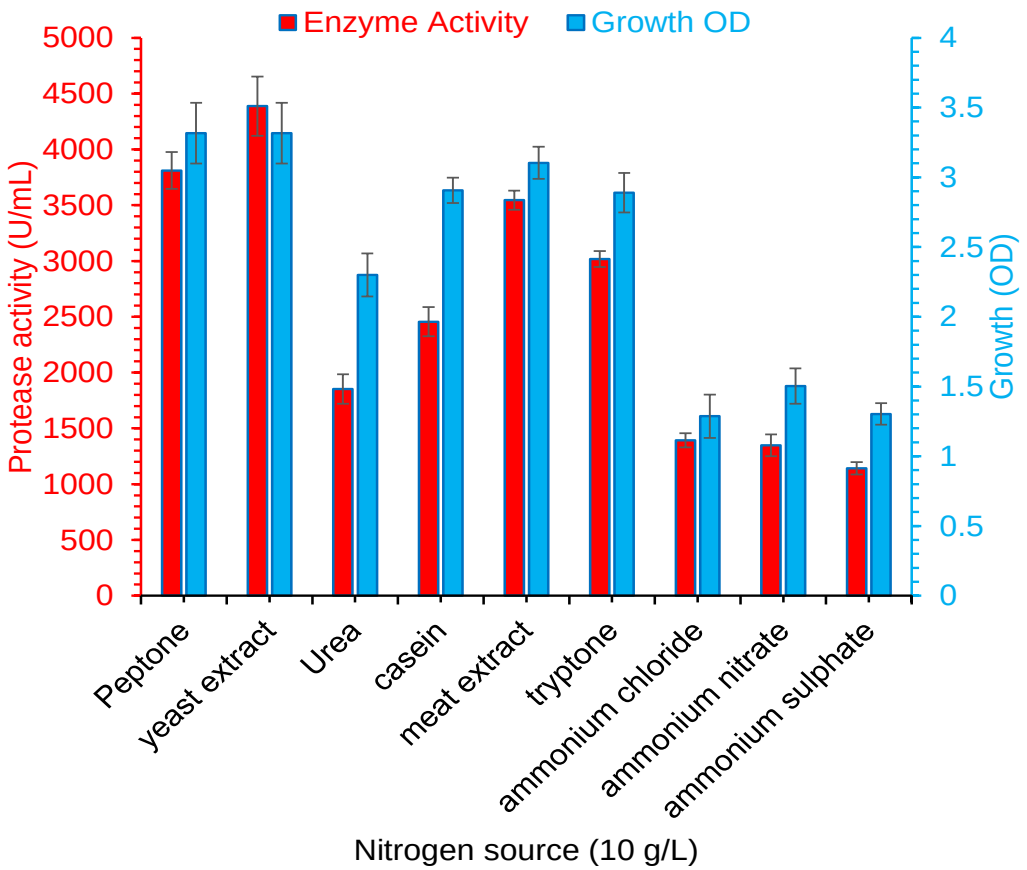

Figure 3. Effect of nitrogen source (10 g/L initial concentration) on biosynthesis of protease from thermophilic *Bacillus licheniformis* BCC-02-50. Experiments were performed in a molasses (20 g/L) mineral medium, and culture was incubated at 37 °C for 36 h. The experiments were performed in triplicate and data presented in figures are average of three parallel experiments. Error bars are shown for standard deviation.

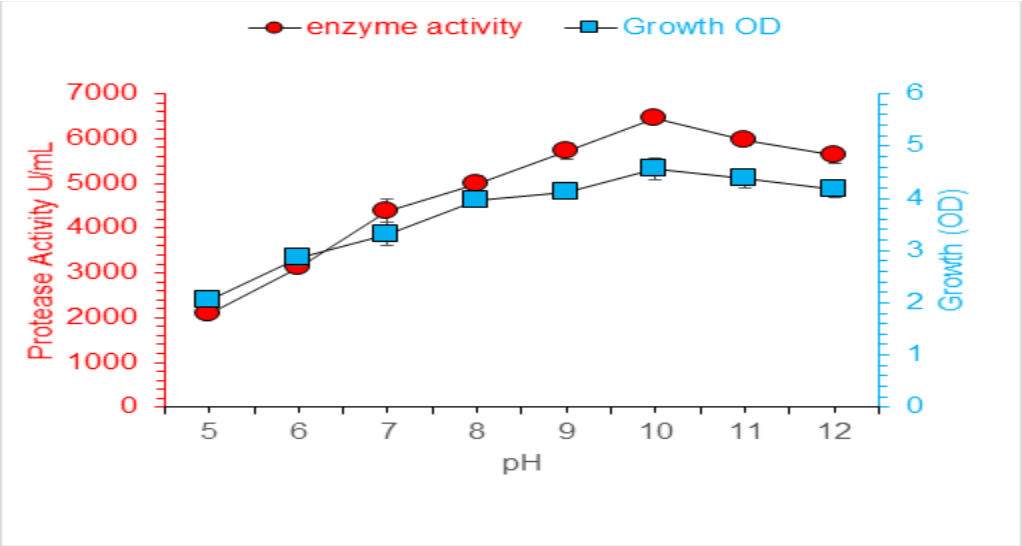

Figure 4. Effect of initial pH on protease production at 37 °C for 36 h in a mineral medium containing molasses (20 g/L) and yeast extract (10 g/L) as carbon and nitrogen sources, respectively. The experiments were performed in triplicate and data presented in figures are average of three parallel experiments. Error bars are shown for standard deviation.

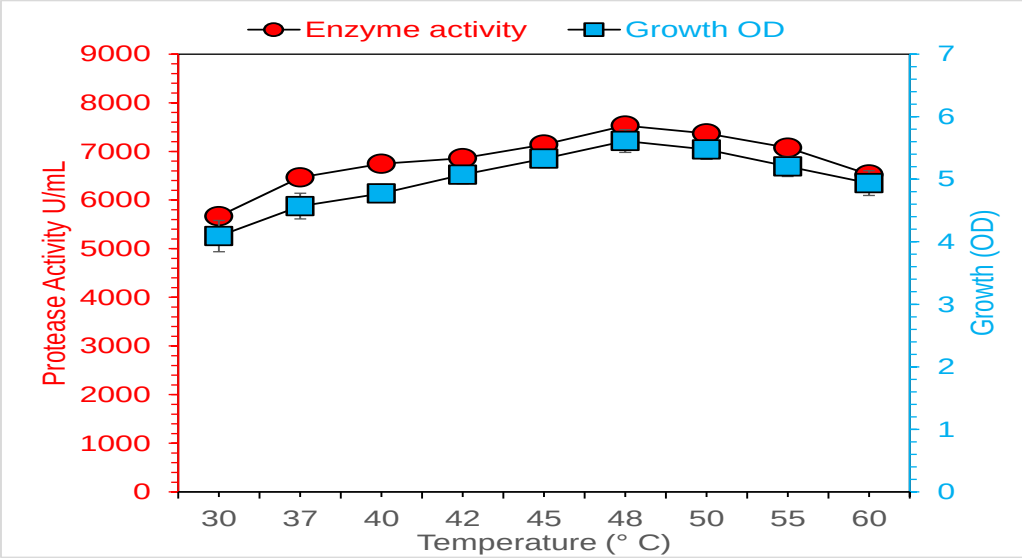

Figure 5. Effect of fermentation temperature on protease production for 36 h. The medium initially contained glucose (20 g/L) and yeast extract (10 g/L) as carbon and

nitrogen sources, respectively. The initial pH was 10. The experiments were performed in triplicate and data presented in figures are average of three parallel experiments. Error bars are shown for standard deviation.

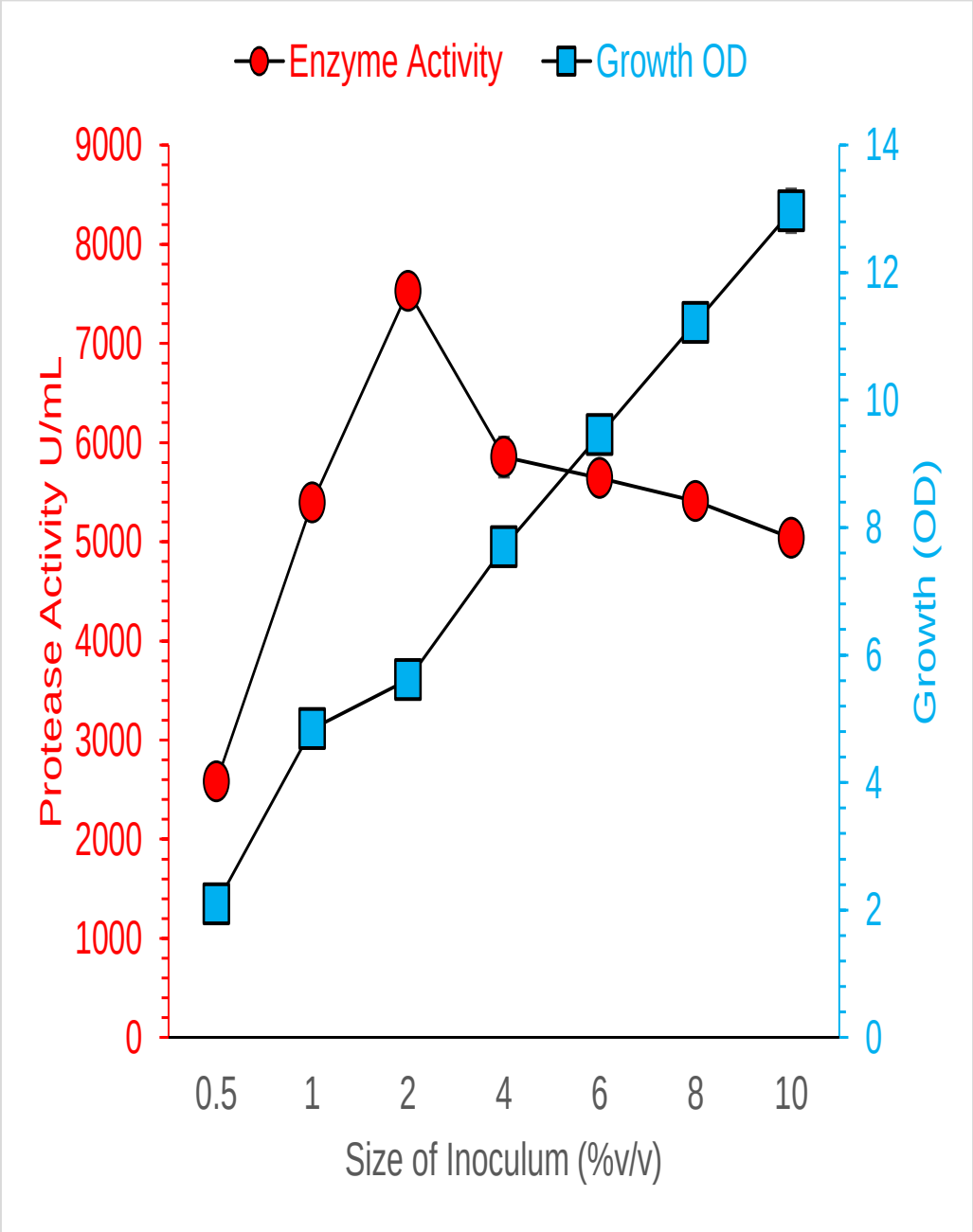

Figure 6. Effect of size of inoculum on protease production at 48 °C for 36 h in a mineral medium containing molasses (20 g/L) and yeast extract (10 g/L) as carbon and nitrogen sources, respectively. The experiments were performed in triplicate and data presented in figures are average of three parallel experiments. Error bars are shown for standard deviation.

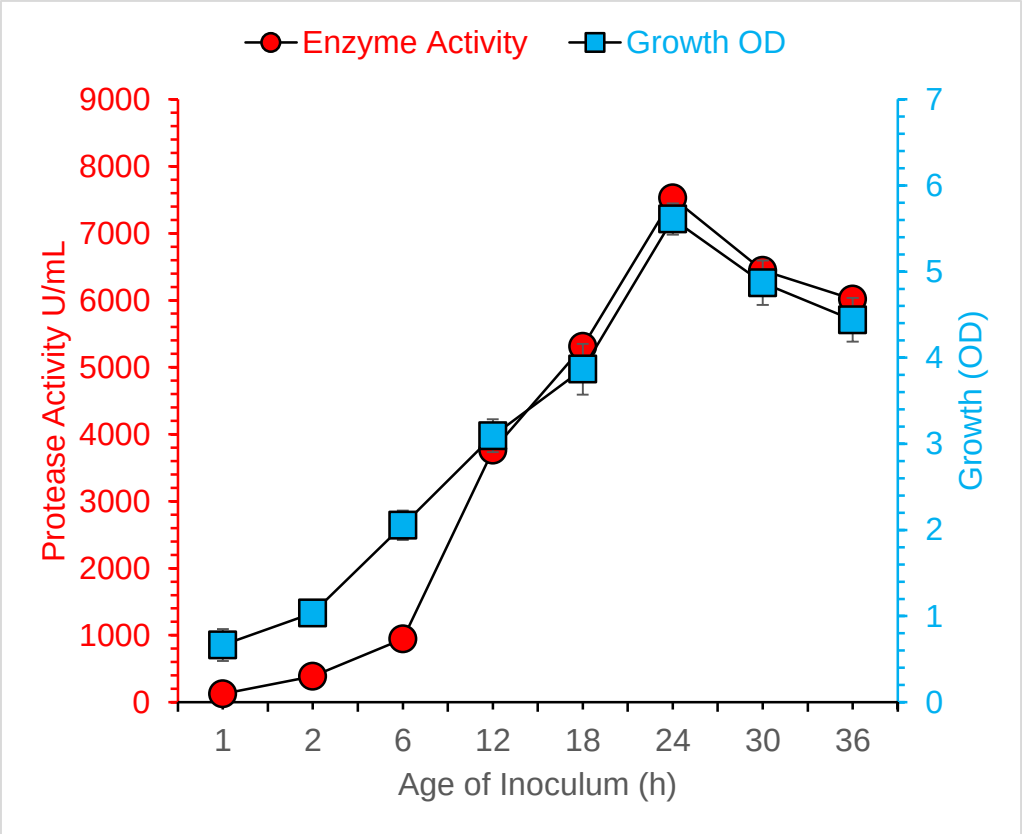

Figure 7: Effect of age of inoculum on protease production at 48 °C for 36 h in a mineral medium containing molasses (20 g/L) and yeast extract (10 g/L) as carbon and nitrogen sources, respectively. The experiments were performed in triplicate and data presented in figures are average of three parallel experiments. Error bars are shown for standard deviation.