Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 4, Suppl 1, 2012

Research Article

SOLID STATE FERMENTATION FOR THE PRODUCTION OF ALKALINE PROTEASE BY *BACILLUS SUBTILIS* KHS-1 (MTCC NO-10110) USING DIFFERENT AGRO- INDUSTRIAL RESIDUES

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Received: 1 Nov 2011, Revised and Accepted: 16 Dec 2011

ABSTRACT

The purpose of this work is to study the production of alkaline protease by *Bacillus Subtilis* (MTCC No-10110) by solid state fermentation (SSF) using different agro- industrial residues (wheat bran, soya bean meal, ground nut cake, green gram husk, coconut oil cake, rice bran saw dust and wheat bran). The optimization of physical parameters such as inoculum size, incubation temperature, initial pH, incubation period and chemical parameters such as additional carbon and nitrogen sources were studied for the production of alkaline protease in solid-state fermentation. The optimum values of the critical components determined for the maximum alkaline protease production were green gram husk (12.5g), glucose (1%w/v), casein (1% w/v) with optimal conditions pH 9.0, temperature 37°C and incubation period 72.

Keywords: Alkaline protease, *Bacillus subtilis*, Optimization, Solid state fermentation

INTRODUCTION

Alkaline proteases, an important group of industrial enzymes, are produced by a wide range of microorganisms, including fungi and bacteria. Bacillus sp. have been reported to produce alkaline protease from various substrates, viz. wheat bran [1-2] soya bean meal [3-4] Rice husk [5-6] ground nut cake [7], green gram husk [8], coconut oil cake [9] and rice bran. *Bacillus* strains have the ability to secrete industrially significant proteases which are stable and compatible with various detergent components [10-11]. *Bacillus licheniformis* has been extensively used in detergent industry for alkaline protease production [12]. However, the cost of alkaline protease is also a major issue in enzyme applications in different industries. About 30–40 % of the cost of industrial enzymes depends on the cost of the growth medium [13]. Carbon and nitrogen sources, inorganic salts and other growth factors are important variables that affect the growth and protease production [14-15]. Proper screenings of the medium ingredients are the basic need of any fermentation process to make it cost-effective and economically feasible at commercial scale. Considering these facts, we have attempted low-cost and easily available medium ingredients for the maximum yield of alkaline protease. Proteases execute a large variety of functions and have numerous applications in detergent, food, pharmaceutical and leather industries [16]. The largest application of the proteases is in the laundry detergents, where they help in removing protein-based stains from clothing during washing. The enzymes to be used as detergent additives should be stable and active in the presence of typical detergent ingredients, such as surfactants, bleaching agents, fillers, fabric softeners and various other formulations [17]. The application of SSF process has a considerable economical potential in the food, feed, pharmaceutical and agricultural industries. In the present study *Bacillus subtilis* KHS-1 (MTCC NO-10110) isolated from slaughter house soil sample [18] was used for the alkaline protease production by solid state fermentation.

Solid substrate medium

Proper screening of the medium ingredients is the basic need of any fermentation process, to make it cost-effective and economically feasible. SSF offers many advantages, including superior productivity, use of inexpensive substrates and simpler downstream processing.

Five grams of different solid substrates (wheat bran, soya bean meal, ground nut cake, green gram husk, coconut oil cake, rice bran saw dust and wheat bran) and 1.5 ml of salt solution (g/100ml: MgSO4.7H2O, 0.02; CaCl2.2H2O,0.05; MnSO4,0.0001) were taken in 250 ml Erlenmeyer flasks and moistened with distilled water, so that substrate moisture content was 70%. Then the contents were mixed thoroughly, autoclaved at 121^{0}C for 30 min and cooled to room temperature. After cooling, flasks were inoculated with 100µl of KHS-1 overnight culture (1X 10^8 cells/ml) and incubated at 37^{0}C per 48 h. Residual activity was measured at every 12 h.

Determination of optimum concentration of best solid substrate

Among different solid substrates used, medium with green gram husk has shown high protease production. To determine the optimum green gram husk concentration, media with different green gram husk concentrations were inoculated with 100 µl and were inoculated with *Bacillus subtilis* KHS-1 overnight culture (1X 10^8 cells/ml) and incubated at 37^0 C for 48 h on a rotary shaker. Samples were withdrawn periodically at every 12 h and observed for protease assay.

Optimization of the culture condition for alkaline protease production

The different physicochemical parameters to maximize the yield of protease by *Bacillus subtilis* KHS-1 under solid state fermentation were investigated. The optimized parameter was incorporated at its optimized level in the subsequent optimization experiments. The impact of

initial moisture content (50-90%), initial pH 7-12 (adjusted with 1N HCl or 1N NaOH), incubation temperature (30-70°C), incubation period (24-1144 h) and size of inoculum on protease production using solid state fermentation *of Bacillus subtilis* KHS-1 was evaluated. Moreover, the effect of incorporation of additional carbon sources (glucose, arabinose, mannitol, sorbitol, and fructose at 1%w/v), additional nitrogenous compounds (yeast extract, casein, beef extract, tryptone and peptone at 1% w/v), to the production medium were studied. All the experiments were conducted in triplicate and the mean values are considered.

Extraction and recovery of enzyme

Protease was extracted from the media using distilled water as extractant. Ten volumes of distilled water per gram substrate were added to the flasks and extraction was performed by agitation at room temperature on a rotary shaker at 220 rpm for 30 min. The slurry was then centrifuged at 10,000 rpm at 4^{0}C for 15 min. The clear supernatant was used as a enzyme source and assayed for protease activity and protein content.

RESULTS AND DISCUSSION

As shown in the Fig. 1, Among 8 different solid substrates used, maximum protease production was observed by green gram husk [19-20] (4764 U/g Biomass) fallowed by soya bean meal (4514 U/g Biomass), wheat bran (4416 U/g Biomass), rice bran (4268 U/g Biomass), ground cake (3845 U/g Biomass), coconut nut cake (2756 U/g Biomass), rice husk (3625 U/g Biomass) and maize bran (3348 U/g Biomass). The medium with green gram husk might contains the protein components and mineral nutrients required for the growth of the bacterium, thereby enhanced protease production as compared to other solid substrates.

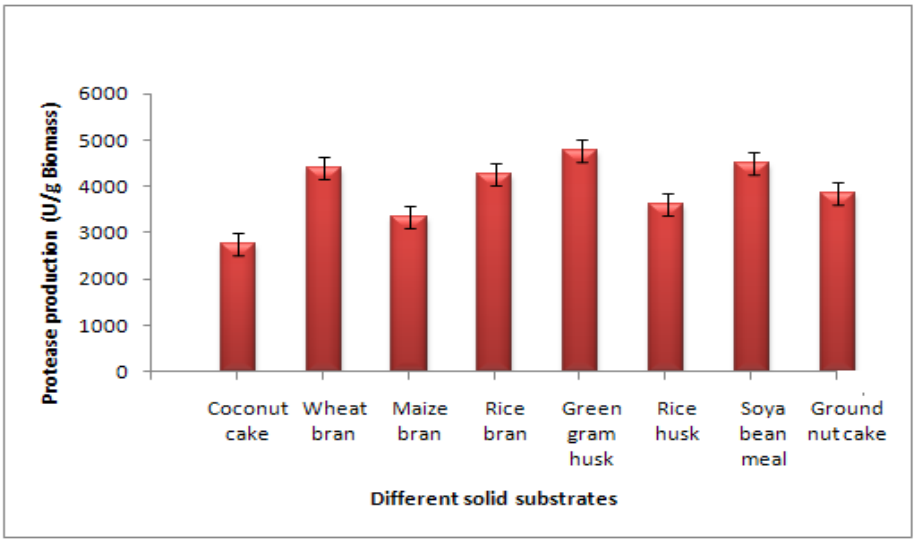


Fig. 1: Protease production using different solid substrates

Effect of different green husk concentration on protease production

As shown in the Fig. 2, maximum protease production (6596 U/g Biomass) was observed at a final concentration of 12.5% green gram husk. Further, increase or decrease of green gram husk concentration above optimal level has decreased protease production**.**

Effect of moisture content on alkaline protease production

The optimal moisture level was found to be 60% for maximum protease production (6645 U/ml). The optimum moisture content for growth and substrate utilization depends on the organism and substrate used for cultivation. The initial moisture content is a critical factor for solid state fermentation processes because this variable has influence on growth, biosynthesis and secreation of different metabolites. The higher moisture level may cause reduction in enzyme yield due to steric hindrance of the growth of strain by reduction in porosity of the solid substrate this interfering with oxygen transfer.

Effect of pH on alkaline protease production

As shown in Fig. 3, Protease was active over a pH range of 8 to 10, with optimum protease production at pH 9.0. The protease production was decreased at above and below optimal pH. Saurab and Praveen kumar also reported maximum protease production by *Bacillus* sp. at pH 9.0 [4-7].

Effect of temperature on protease production by solid state fermentation

As shown in the Fig. 4, optimum protease production (6866 U/ml) was observed at 37^0 C. The protease production was decreased at above and below optimal temperature. Temperature affects microbial cellular growth and microbial physiology, thus affecting product formation in turn.

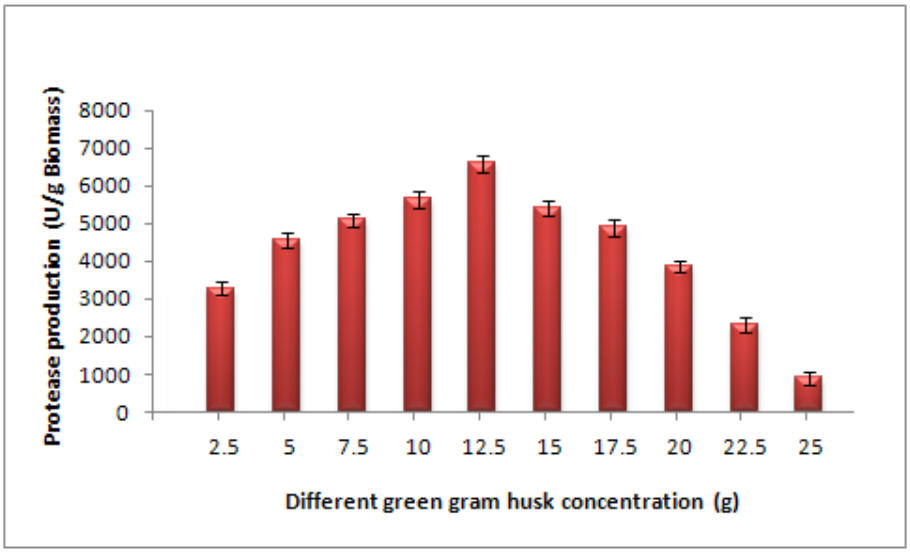
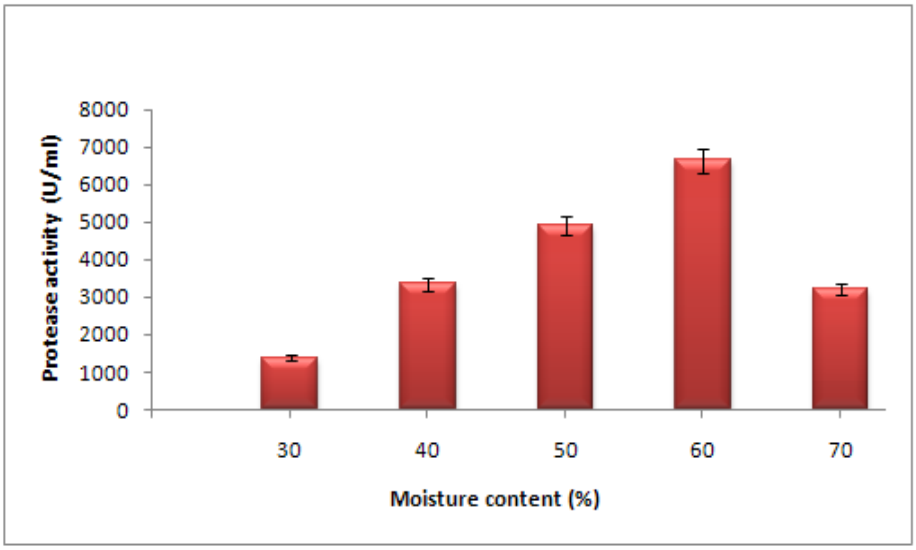


Fig. 2: Effect of green gram husk concentration on protease production



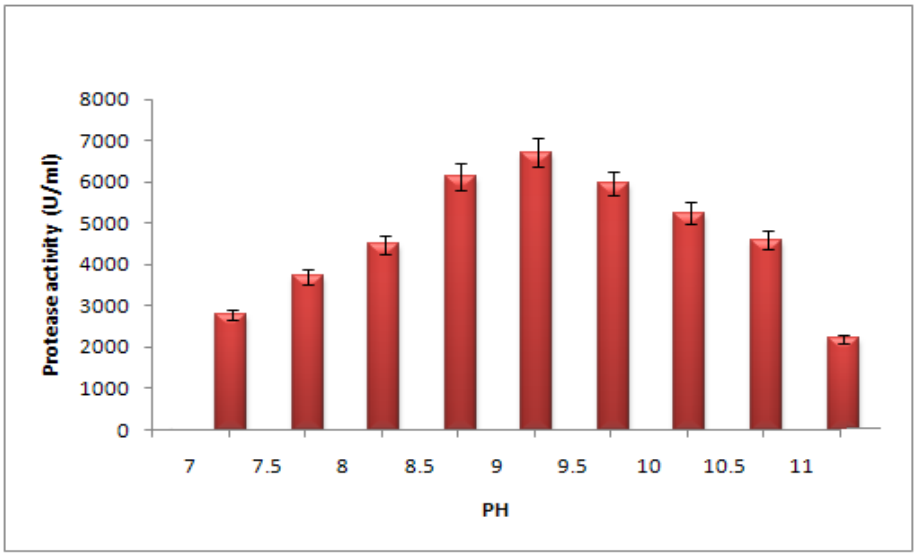


Fig. 3: Effect of pH on alkaline protease production by solid state fermentation

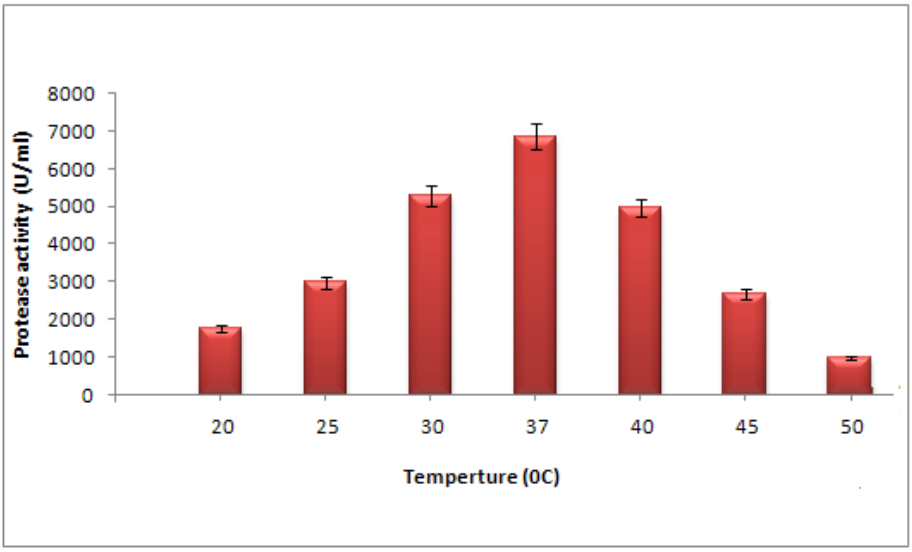


Fig. 4: Effect of temperature on alkaline protease production

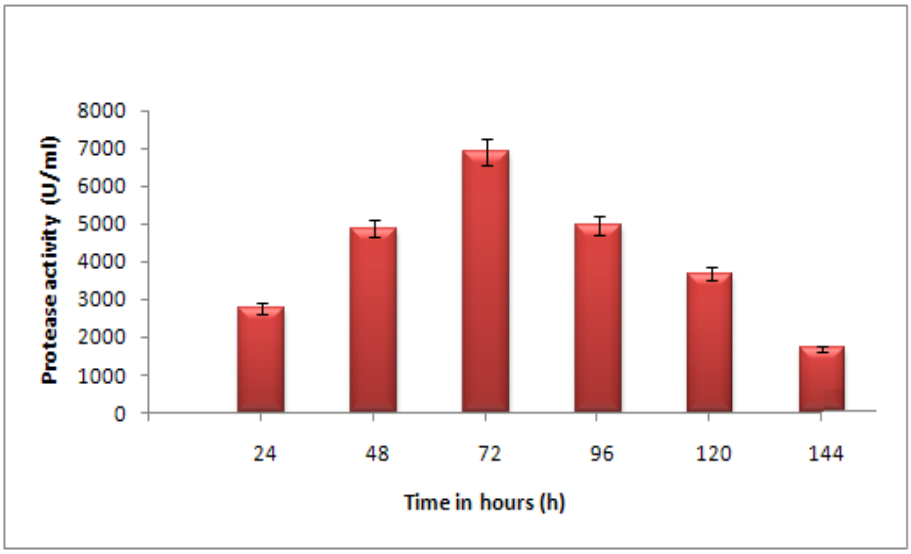


Fig. 5(a): Effect of incubation period on protease production

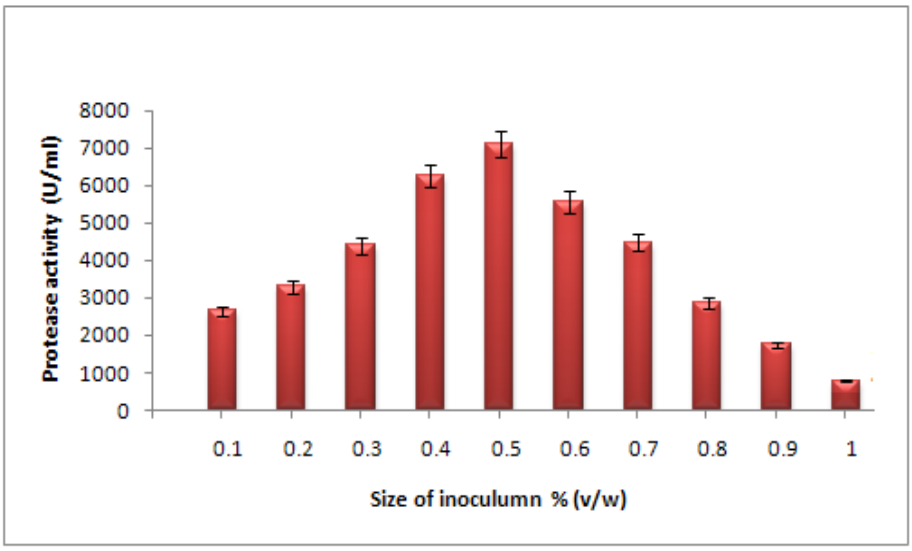


Fig. 5(b): Effect of inoculum size on protease production by solid state fermentation

Effect of incubation period on protease production

As shown in the Fig. 5(a), maximum protease production was observed (6928 U/ml) at 72 h of incubation. A gradual decrease in enzyme production was observed with increasing incubation period clearly suggesting the enzyme's role as a primary metabolite, being produced in the log phase of the growth of Bacillus subtilis KHS-1 for utilization of nutrients (proteins) present in the solid substrate. Sumantha Alagarsamy *et al.,* (2006)[21] and Paranthaman (2009) [6] also reported maximum protease production by *Rhizopus sp.* and *Aspergillus niger* at 72 h incubation.

Effect of inoculum size on protease production

As shown in the Fig. 5(b), the optimum protease production (7096 U/ml) was observed at 0.5 ml (v/v) inoculum size. A decrease in protease production was observed when the inoculum size was increased beyond the optimum level. Protease production attains its peak when sufficient nutrients are available to the biomass. Conditions with a misbalance between nutrients and proliferating biomass result in decreased protease synthesis.

Effect of carbon source on protease production

Although green gram husk supports the growth of *Bacillus subtilis* KHS-1 and protease production, it may not provide enough carbon sources needed by the organism for maximum protease production. Hence, the exogenous addition of various carbon sources to the medium has enhanced the protease production. As shown in Fig. 6, among all carbon sources, maximum protease production was observed with glucose (8684 U/ml) fallowed by Arabinose (8098 U/ml), fructose (7445 U/ml), sucrose (6894 U/ml) and lactose (6128 U/ml).

Effect of nitrogen source on protease production

The protein content in green gram husk is very low so that the nitrogen levels as well as the commercial value all decrease greatly. Hence, the exogenous addition of various nitrogen levels to the solid medium was studied. As shown in Fig. 7, among different nitrogen sources, maximum protease production was observed with casein (8848 U/ml), fallowed by yeast extract (7942 U/ml), beef extract (7288 U/ml), peptone (6584 U/ml) and tryptone (3386 U/ml).

As shown in the Fig. 8, optimized production medium and production conditions enhanced the alkaline protease production by *Bacillus subtilis* KHS-1 by 199%.

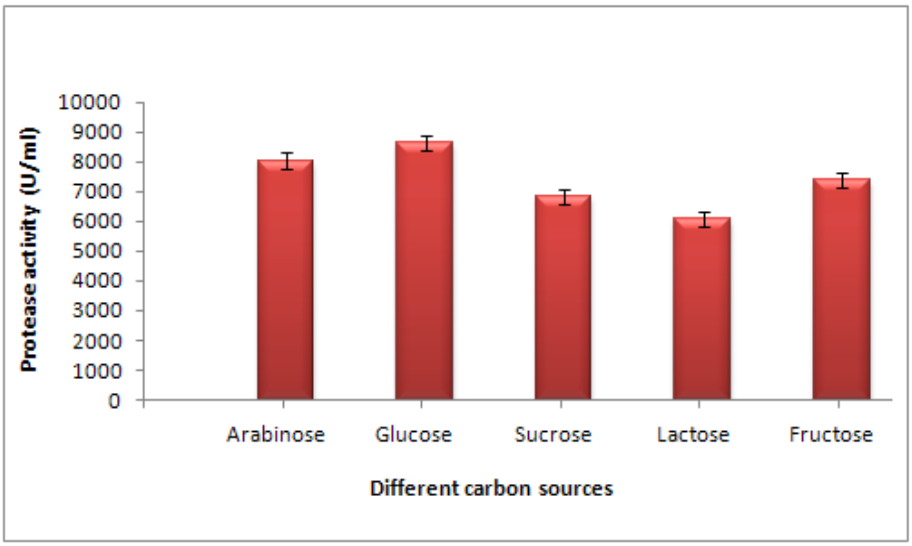


Fig. 6: Effect of carbon source on protease production

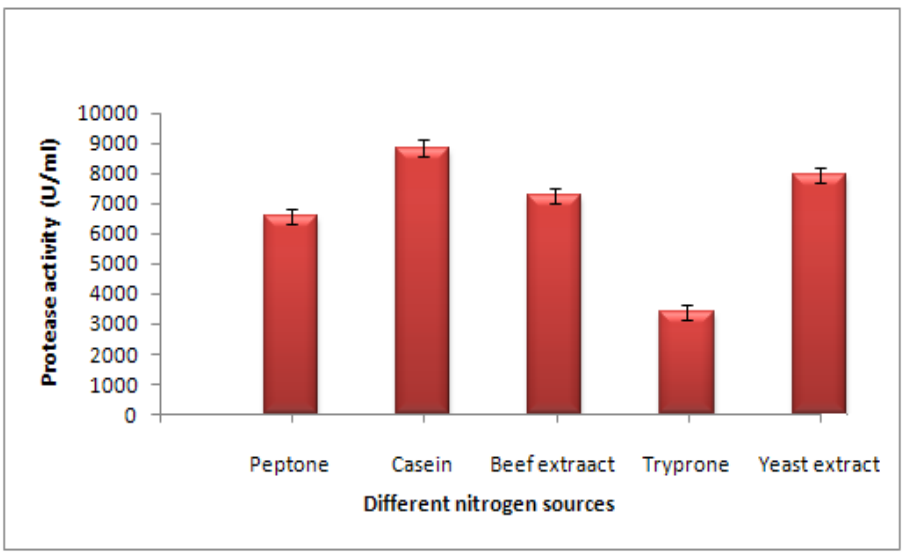
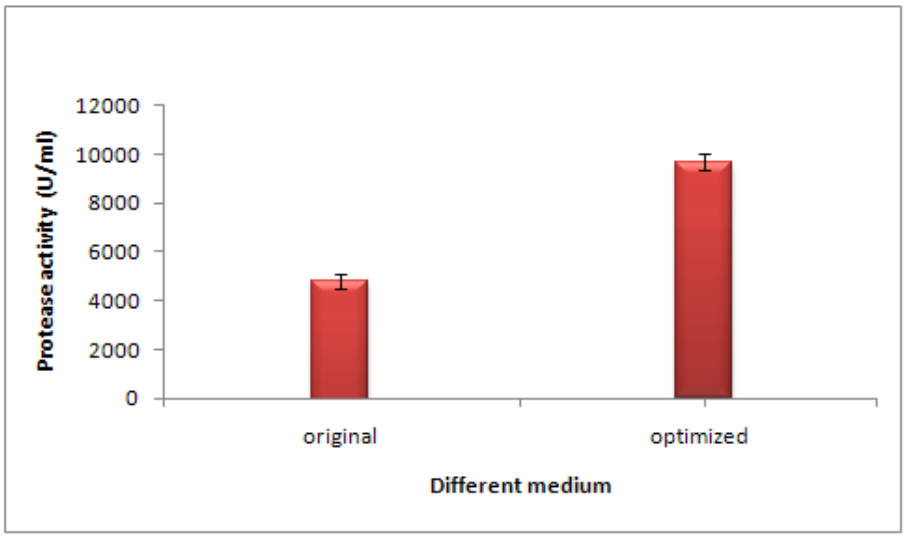


Fig. 7: Effect of carbon source on protease production



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