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ORIGINAL ARTICLE

Optimization and enhanced production of α-amylase and protease by a newly isolated *Bacillus licheniformis* ZB-05 under solid-state fermentation

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Abstract Eight different agro-residues were tested for α-amylase and protease production by using *Bacillus licheniformis* ZB-05. Among them, rice husk (RH) was proved as the best substrate for two enzymes (α-amylase 443 U/g and protease 469,000 U/g). Maximum enzyme production was observed to be 30 % initial moisture, with a growth period of 36 h in 20 and 30 % inoculum volumes for α-amylase and protease, respectively. The best enzyme recovery from solid mass was obtained when extracted with tap water. Among the tested various nitrogen sources, 1 % ammonium sulphate followed by 2 % Bacto liver, 2 % ammonium sulphate and 1 % Bacto casaminoacid served as the best inorganic and organic nitrogen sources for α-amylase and protease production, respectively. As additional carbon sources, 2 % soluble starch enhanced α-amylase production, while 1 % maltose enhanced protease production.

Keywords α-amylase · Agricultural waste · *Bacillus licheniformis* · Enzyme production · Protease · Solid-state fermentation

Introduction

The development of enzyme technology has raised the value of industrial enzymes used in biotechnology. These enzymes can be obtained from various sources such as plants, animals and microorganisms, but generally, microbial enzymes meet industrial demands (Mahanta et al. 2008; Merheb Carolina et al. 2007). Amylases and proteases are two of these industrial enzymes.

α-Amylases are enzymes (α, β glucoamylase) which are widely used in industry for starch hydrolysis. α-Amylases are extracellular enzymes that randomly cleave the α-1,4 glucosidic bonding of linear amylose and branching amylopectin. These enzymes are used in the paper, food, pharmaceutical and sugar industries. They are also used in starch liquefaction and sugar syrup production from starch (Hagihara et al. 2001; Xu et al. 2008). The fermentable carbon sources such as glucose, starch, lactose or fructose are obtained after the processing of agricultural products. Therefore, they became very expensive for commercial production of α-amylase. These expensive products can be replaced in the fermentation medium with economically available agricultural by-products (Haq et al. 2005; Uyar et al. 2003).

Protease refers to a group of enzymes whose catalytic function is to hydrolyze peptide bonds of proteins. They are also called proteolytic enzymes or proteinases. Each type of protease has a specific kind of peptide bonds that it breaks. These enzymes play an important role in the decomposition of vegetative, animal and microbial residues in nature. Proteases represent one of the largest groups of industrial enzymes which have increasing market demands due to their applications in the detergent, food, pharmaceuticals, chemicals, leather, paper, pulp and silk industries (Mukherjee et al. 2008; Gupta et al. 2002; Esakkiraj et al. 2011). Microorganisms have long played a major role in the production of food (dairy, fish and meat products) and of alcoholic beverages. In addition, several products of microbial fermentation are also incorporated into food as additives and supplements (antioxidants, flavors, colorants, preservatives, sweeteners) (Rodriguez Couto and Angeles Sanroman 2006).

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Traditionally, enzymes have been produced by SmF (submerged fermentation). However, in recent years, SSF (solid-state fermentation) processes have increasingly been utilized for the production of enzymes. SSF is characterized by the growth of microorganisms within particles of a solid substrate that act as a source of carbon, nitrogen, minerals, and growth factors, and has a capacity to absorb water, necessary for microbial growth in the presence of varying amounts of water (Han et al. 1999; Pandey 2003; Goes and Sheppard 1999). A further advantage of SSF is that they are cheap and easily available substrates, such as agriculture and food industry by-products (Tanyildizi et al. 2007). Bacteria, yeasts and fungi can grow on solid substrates and find applications in SSF processes (Pandey et al. 2000a, b, c; Sodhi et al. 2005).

Enzyme production with SSF would be more eco-friendly and economical than SmF because of less water consumed and lower costs of substrates and equipment. SSF has tremendous potential for the production of enzymes and has been increasingly applied for this purpose in recent years (Pandey et al. 2000a, b, c; Sangeetha et al. 2004).

Therefore, the present study represents an investigation into α-amylase and protease production by SSF with some agricultural wastes as substrates, and the determination of optimized production conditions.

Materials and methods

Selection and isolation of bacterial strain

Bacillus licheniformis, a moderate thermophilic bacterium, was isolated from the Van Lake coast by Dr. Zübeyde Baysal. Culture suspension in sterilized water was spread on nutrient agar. The plate was incubated at 37 °C for 24 h. A sample from positive growth on nutrient agar medium was selected. The identification was done with 16S ribosomal RNA by Refgen Biotechnology Center, Middle East Technical University.

Inoculum preparation

A volume of 50 ml of nutrient broth taken in a 250-ml Erlenmeyer flask was inoculated with a loopfull of cells from a 24-h-old plate and kept at 37 °C in a rotary shaker. After 24 h of incubation, 1 ml of this nutrient broth culture was used as the inoculum. By serial dilution and plating, the number of viable colonies in the inoculum was found to be 3×10^6 CFU/ml.

Fermentation medium

Rice husk (RH), wheat bran (WB), maize oil cake (MOC), millet (M), lentil bran (LB), orange peel (OP), banana peel (BP) and apple peel (AP) were obtained from local market in Diyarbakır, Turkey. These agro-industrial products were used as solid substrates, and their effect on the production of α-amylase and protease was determined. The best solid substrate selected and used in subsequent experiments.

Three grams of solid substrate, which passed through sieve of 1,500 μm, were taken into 100-ml conical flasks. The substrates were moistened with tap water. After autoclaving at 121 °C for 15 min, and cooling to room temperature, the flasks were inoculated with 0.5 ml spore suspension and incubated at 37 °C at 200 rpm.

Enzyme extraction

Enzyme extraction was performed according to the method of Ramachandran et al. (2004). Crude enzymes were extracted by mixing a known given amount of fermented substrate with tap water and then shaking at 200 rpm. The suspension was centrifuged at 5,000 *g* for 5 min, and then the clear supernatant was used for α-amylase and protease assay.

α-Amylase activity

α-Amylase activity was determined as described by Bernfeld (1955). An amount of 150 μl enzyme solution was added to 200 μl of 0.2 % soluble starch (w/v) solution in 0.1 M Tris–HCl buffer (pH 7.0) and the mixtures incubated at 37 °C for 30 min. The reaction was stopped by addition of 400 μl 3,5 dinitro salicylic acid and was kept in boiling water for 5 min. The mixtures were held until reaching room temperature and then diluted by adding 8 ml distilled water. The absorbance of samples was measured at 489 nm.

One unit of enzyme activity is defined as the amount of enzyme which releases 1 μmol of reducing end groups per minute in 0.1 M Tris–HCl buffer (pH 7.0) with 0.2 % soluble starch as substrate at 37 °C.

Protease activity

Protease activity was determined by using sulphanilamide azocasein substrate according to the method of Leighton (Leighton et al. 1973). The reaction mixture containing 250 μl 0.5 % azocasein (w/v) in 0.1 M phosphate buffer (pH 8.5) and 150 μl of enzyme solution was incubated for 30 min at 37 °C. After incubation, the enzyme was inactivated by addition of 1.2 ml trichloroacetic acid solution (10 %, w/v) and then the solution was neutralized by adding 800 μl of 1.8 N NaOH solution. The absorbance was read at 420 nm.

One unit of proteolytic enzyme activity was defined as the amount of azocasein that was hydrolyzed during 1 h incubation at 37 °C for 1 ml of solution of extract.

All the experiments were performed in triplicate and the standard error was expressed.

Optimization of medium and culture conditions

Various process parameters influencing enzyme production during SSF were optimized. The strategy followed was to optimize each parameter, independent of the others, and subsequently optimal conditions were employed in all experiments. In a sequential order, the various process parameters were optimized for maximal enzyme production as follows: selection of the best substrate (RH, WB, MOC, M, LB, OP, BP and AP), incubation period (12, 24, 36, 48, 60 and 72 h), inoculum volume (5, 10, 15, 20, 25, 30 and 35 %), and moisture level (20, 30, 40, 50 and 60 %). Nutrient supplementation such as nitrogen sources (Bacto casaminoacid, Bacto liver, ammonium sulphate, urea, ammonium chloride and methionin) and carbon sources (soluble starch, glucose, fructose, maltose, galactose and sucrose) were also optimized at 1, 2 and 3 % concentrations. Distilled water, tap water, 2 % SDS, 50 mM NaCl, 50 mM acetate buffer (pH 6.0, for α-amylase) and 50 mM phosphate buffer (pH 8.5, for protease) were used independently to final the best extraction medium for the enzymes.

Statistical analysis

All experiments were carried out in triplicate and the results are expressed as means ± standard deviation (SD). Statistical significance was determined by analysis of variance (ANOVA) using SPSS software version 14.0 (Illinois, USA). Significant of means were determined using the Tukey and Games-Howell post hoc test at a significant difference ($p<0.01$).

Results and discussion

Screening of agro-industrials as substrate for SSF

α-Amylase and protease production by *B. licheniformis* ZB-05, under SSF using different solid substrates were optimized for various physicochemical parameters. Among the various substrates screened for SSF, such as WB, RH, MOC, M, LB, OP, BP and AP, RH yielded the highest enzyme activity (443 and 469,000 U/g for α-amylase and protease, respectively) at 36 h (Figs. 1, 2). RH and WB have widely been reported to be the best substrates for α-amylase and protease production in SSF due to their rich contents such as vitamins and minerals (Ellaiah et al. 2002; Agrawal et al. 2005).

Effect of incubation period on enzyme production

The incubation time course of α-amylase and protease production from *B. licheniformis* using RH as a substrate is shown in Fig. 2. The α-amylase and protease production were found to be maximum (400 and 341,000 U/g, respectively) at 36 h and decreased thereafter. The reason for this might have been due to the depletion of the nutrients or denaturation of the enzymes caused by the interaction with other components in the medium or change in the pH of the medium (Krishna and Chandrasekaran 1996).

Kaur et al. (2001) observed maximum protease yields between 120 and 168 h of incubation at 30 °C in SSF medium by *Bacillus* sp. P-2. α-Amylase production was

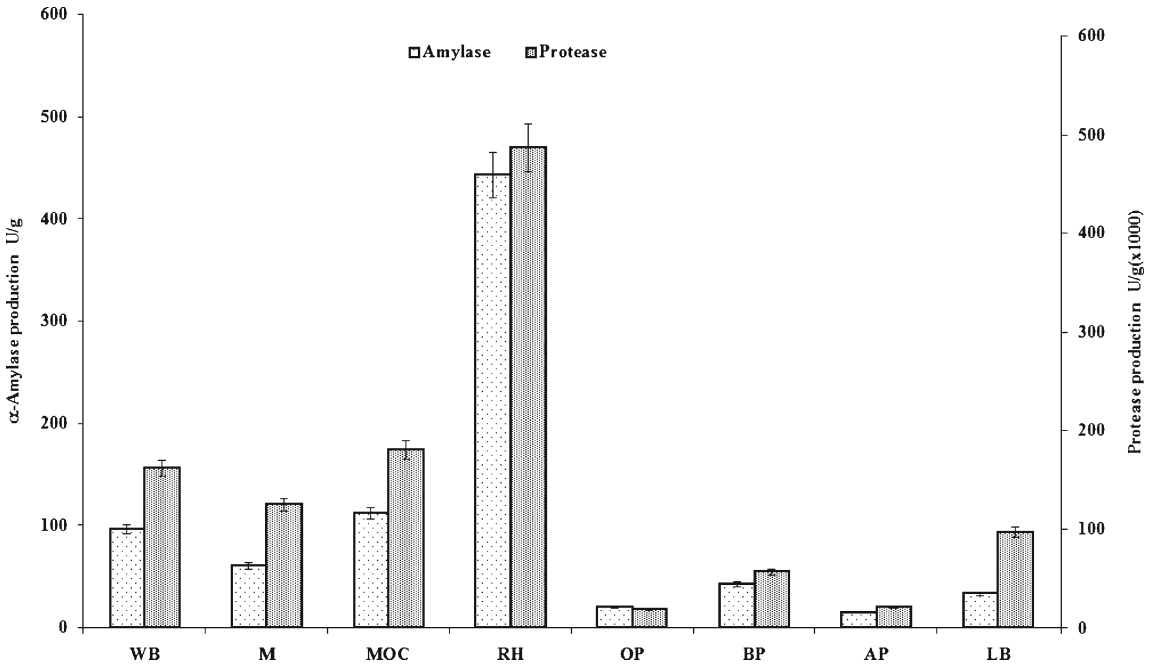

Fig. 1 Screening of agro-industrials as substrate for α-amylase and protease production. (For both α-amylase and protease, *RH* represents significantly different results at $p<0.01$)

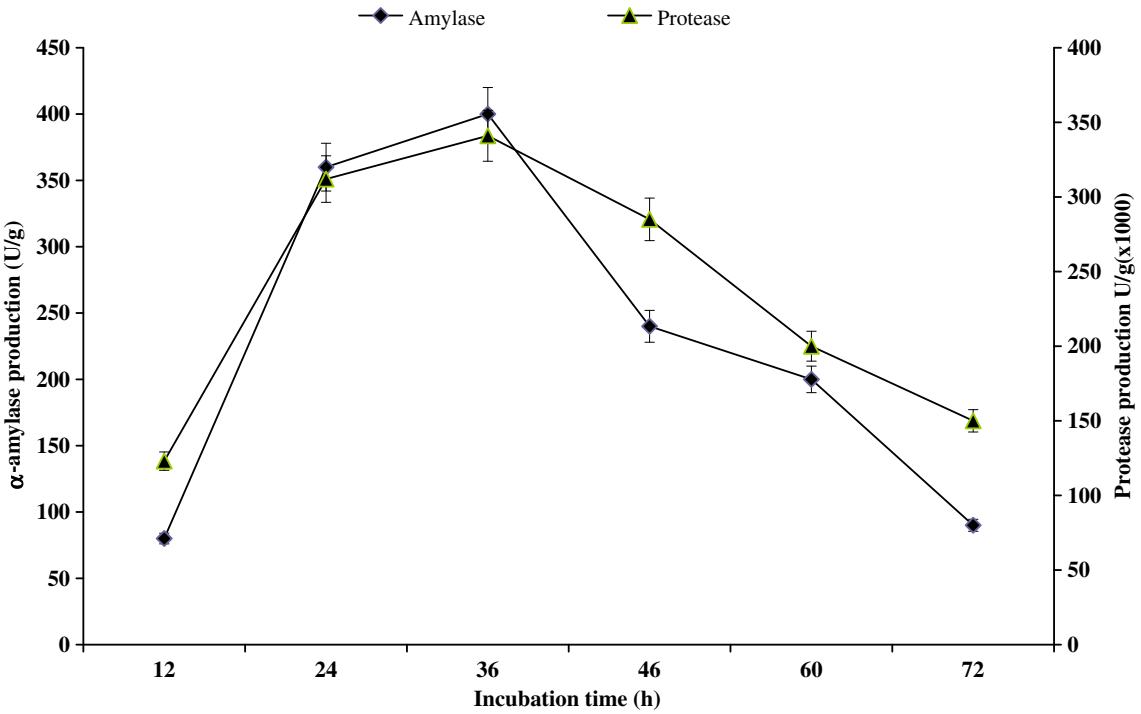

Fig. 2 Effect of inoculum volume on α-amylase and protease production (15 and 20 % inoculum volume for α-amylase and, 25 and 30 % for protease represents significantly different results at $p<0.01$)

found to be 48 and 24 h with WB and RH, respectively (Baysal et al. 2003). It has also been suggested that α-amylase has usually yielded highest activity at 24 h (Baysal et al. 2003; Uyar and Baysal 2004).

Effect of inoculum volume on enzyme production

As shown in Fig. 3, an increase in inoculum volume from 10 to 35 % (v/w) α-amylase and protease production was obtained. Maximum α-amylase and protease production were observed with 20 and 30 % inoculum volumes, respectively. With 35 % inoculum concentration, no significant decline was observed in enzymes production. A higher inoculum concentration increased the moisture content to a significant extent. The free excess liquid present in an unabsorbed form will therefore give rise to an additional diffusional barrier, together with that imposed by the solid nature of the substrate, and lead to a decrease in enzyme production (Krishna and Chandrasekaran 1996).

Effect of initial moisture content on α-amylase and protease production

Results summarized in Fig. 4 show that 30 % of moisture was optimal, giving maximum amounts of α-amylase and protease production (660 and 462,000 U/g of substrate, respectively). The moisture content is a critical factor in solid-state fermentation. Its importance for microbial growth and thereby enzyme production has been well established. In fungal and bacterial SSF, lower moisture content was stated to lead to reduced solubility of the nutrients present in the solid substrate, a lower degree of substrate swelling, and higher water tension. Similarly, higher moisture content may cause decreased porosity, loss of particle structure, development of stickiness, and reduction in gas volume (Chellappan et al. 2006). The necessary moisture in SSF exists in absorbed or complex form within the solid matrix, which is likely to be more advantageous for growth because of the possible efficient oxygen transfer process (Gangadharan et al. 2006).

Results depicted in Fig. 4 show that 30 % of moisture was optimal for α-amylase and protease production. An increase in moisture content causes a decrease in the porosity of the substrate, thereby decreasing the gas exchange. A low moisture content leads to sub-optimal growth and a lower degree of substrate swelling which also decreases enzyme production (Rodriguez Couto and Angeles Sanroman 2006). Earlier reports indicate the requirement of 50 % moisture content to be optimum for maximal protease yield in the case of *Bacillus* sp (Kaur et al. 2001). In the case of *Bacillus amyloliquefaciens*, optimal moisture level was also found to be 85 % for α-amylase production (Gangadharan et al. 2006).

Effect of nitrogen supplementations on enzyme production

Figure 5 shows the effect of nitrogen source in fermentation medium on α-amylase production. In all concentrations, the

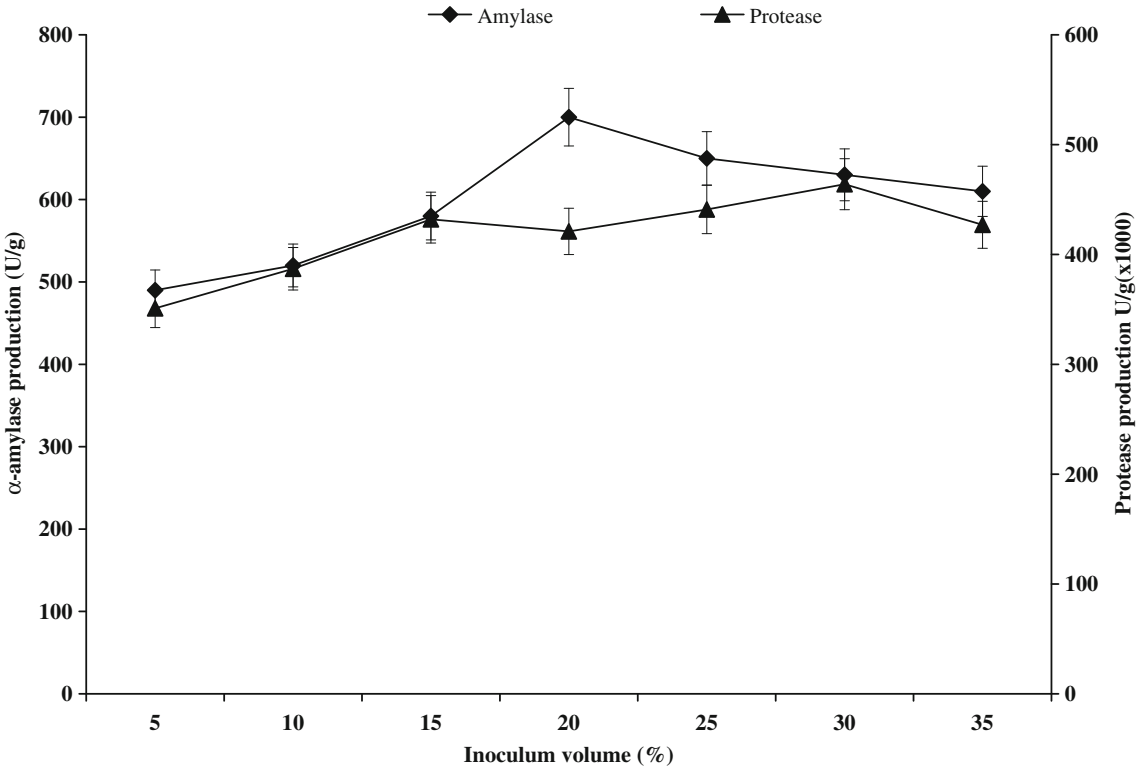

Fig. 3 Effect of incubation period on α-amylase and protease production (For both α-amylase and protease, 24 and 36 h represent significantly different results at $p < 0.01$)

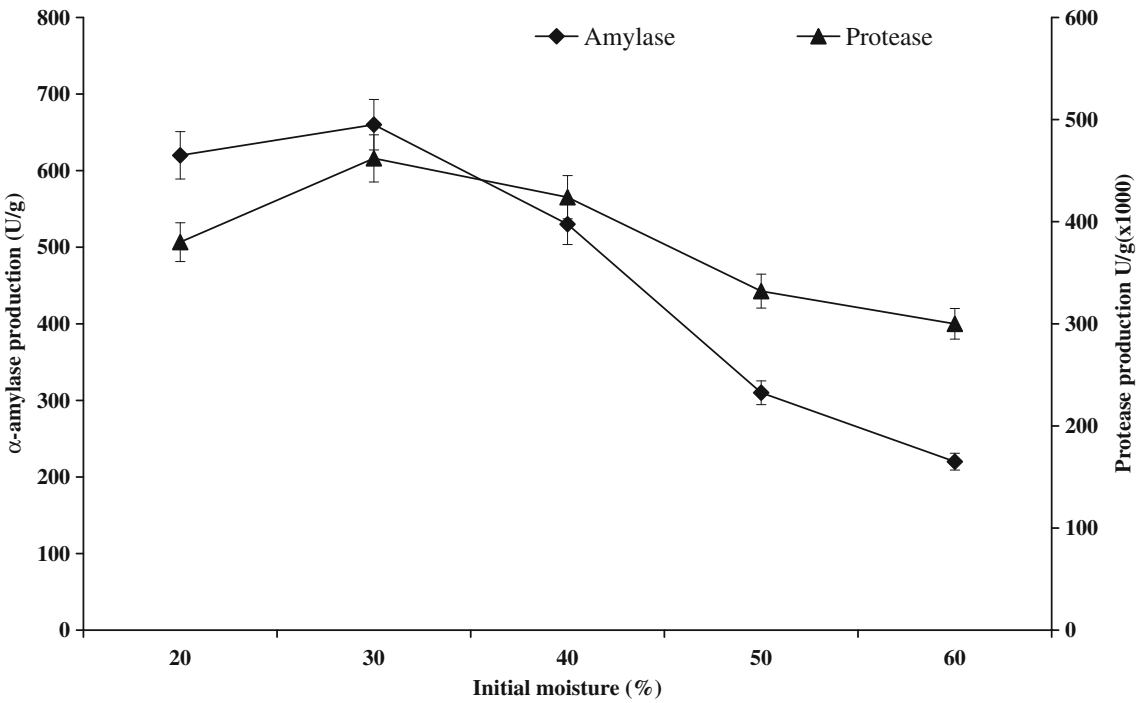

Fig. 4 Influence of initial moisture content of the medium on the production of α-amylase and protease (20, 30, and 40 % initial moisture volume for α-amylase, and 30 and 40 % for protease represent significantly different results at $p<0.01$)

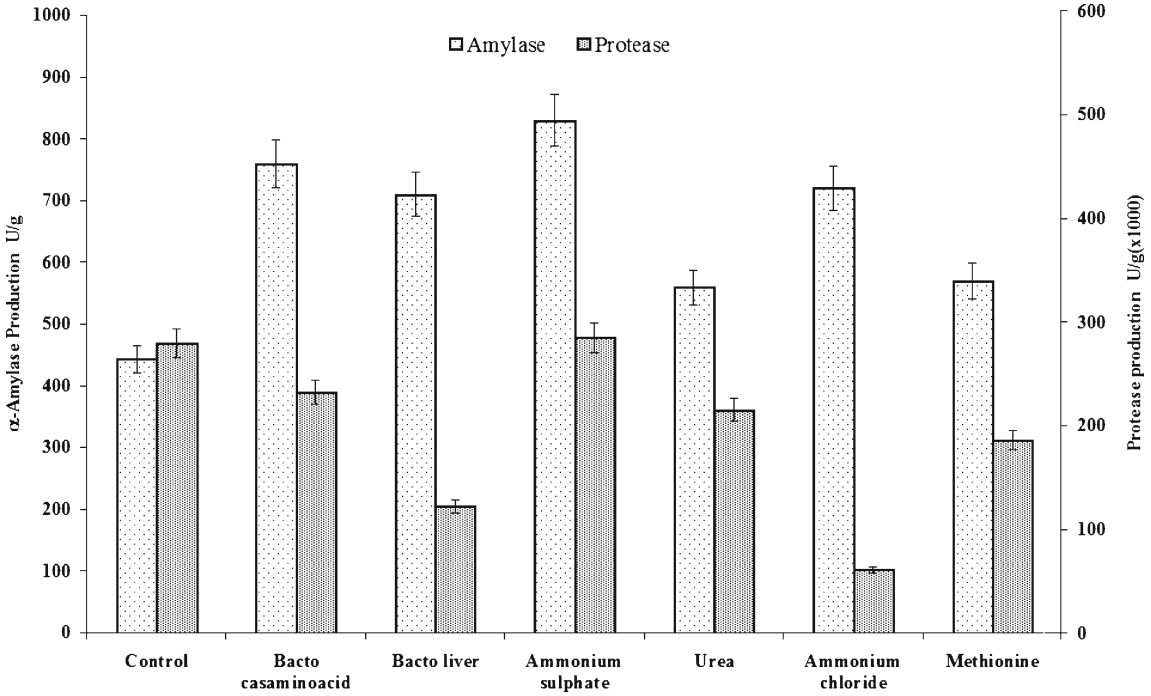

Fig. 5 Effect of nitrogen supplementations on α-amylase production and protease (ammonium sulphate and bacto casaminoacid represent significantly different results at $p<0.01$ for α-amylase but there were no significantly different results for protease)

higher enzyme activity was obtained when 1 % ammonium sulphate was used as a nitrogen source (830 U/g). Added nitrogen sources have been reported to have an inducing effect on the production of various enzymes including α-amylase in an SSF system (Gangadharan et al. 2006). Earlier reports show that, among various inorganic nitrogen sources

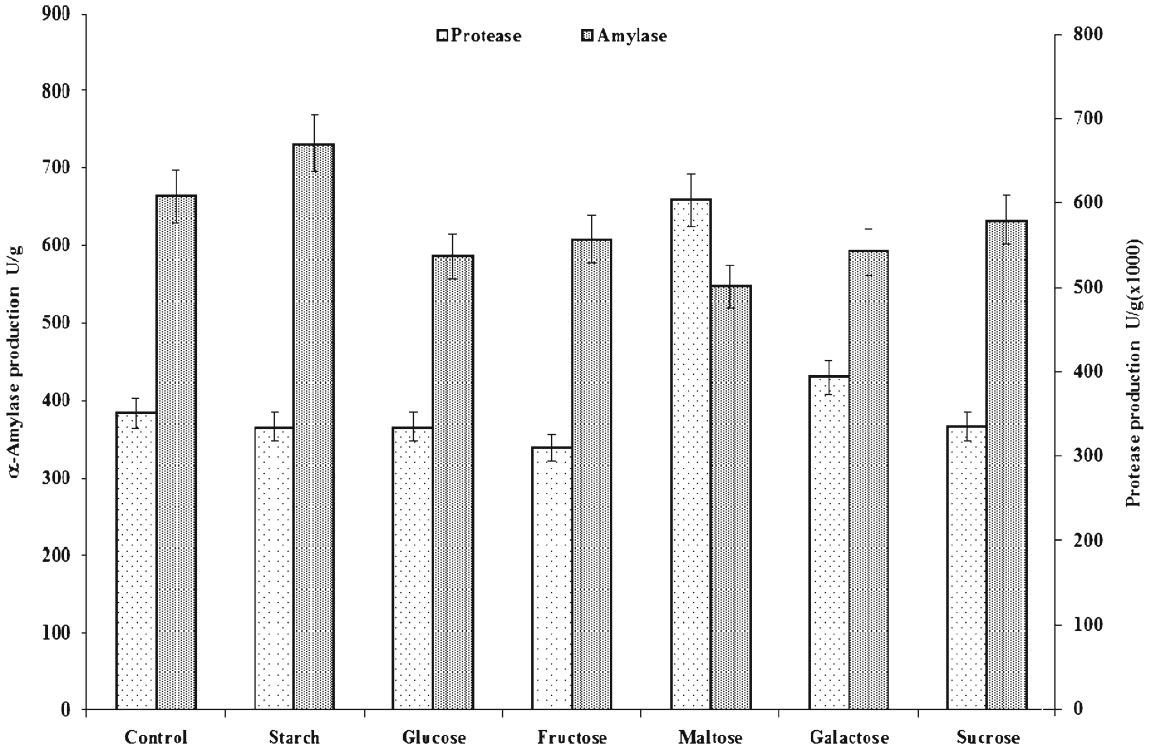

Fig. 6 Effect of carbon supplementations on α-amylase production and protease. (For α-amylase starch and maltose for protease represents significantly different results at $p<0.01$)

Table 1 Effect of extraction solvents on the production of α-amylase and protease by *B. licheniformis* (Means ± standard deviation)

Extraction solvents	α-amylase activity (U/g)	Protease activity (U/g × 1,000)
Distilled water	25±2.8	56±4.2
Tap water	660±16.7	470±11.3
Buffer	90±6.3	96±5.1
SDS 0.1%	15±1.0	125±7.3
NaCl 50 mM	Not detected	60±4.0

tested, ammonium sulphate, ammonium chloride and ammonium hydrogen phosphate favored growth and enzyme secretion (Narang and Satyanarayana 2001). The effect of nitrogen source on α-amylase production, in all concentrations, is indicated in Fig. 5, and the highest enzyme activity was obtained with 1 % ammonium sulphate. One of the complex nitrogen sources, Bacto liver (2 %), led to an increase in enzyme production. Haq et al. (2005) have shown that 1 % nutrient broth favored α-amylase production by *B. licheniformis*.

Some researchers have suggested that different bacteria have different preferences for either organic or inorganic nitrogen for growth and protease production, although complex nitrogen sources are usually used for alkaline protease production (Prakasham et al. 2006; Pandey et al. 2000a, b, c). Mukherjee et al. (2008) reported that the *Bacillus subtilis* strain used in their study showed a preference for organic nitrogen sources compared to inorganic nitrogen for protease production. However, in our studies, as indicated in Fig. 5, maximal increase was noted with the addition of 2 % ammonium sulphate (478,000 U/g).

Effect of carbon supplementations on enzyme production

Simple and complex carbon sources such soluble starch, glucose, fructose, maltose, galactose and sucrose were used as carbon sources to investigate their effect on α-amylase and protease production (Fig. 6). α-Amylase production was found to be 736 U/g with 2 % soluble starch. In the case of protease, 1 % maltose was observed as the best carbon source (658,000 U/g). The choice of the carbon sources has a major influence on the yield of α-amylase and protease. α-Amylase is an inducible enzyme, which is generally induced in the presence of starch or its hydrolytic product maltose (Gangadharan et al. 2006). Simple and complex carbon sources, such as soluble starch, glucose, fructose, maltose, galactose and sucrose, were used as carbon sources to investigate their effect on α-amylase and protease production (Fig. 6). Easily metabolizable carbohydrates may result in better growth of the bacteria along with reduction in the enzyme formation (Rama and Srivastava 1995). *Bacillus thermoleovorans* preferred starch, glucose, lactose, maltose and maltodextrin as favorable carbon sources for amylase secretion (Narang and Satyanarayana 2001). Amozeegar et al. (2003) observed maximum amylase activity by *Halobacillus* sp. strain MA-2 with dextrin.

In the literature, protease production was found to be enhanced by carbon sources such as maltose and sucrose (Mukherjee et al. 2008; Mahanta et al. 2008; Chellappan et al. 2006).

Effect of various extraction medium on the enzyme production

The medium used for the extraction of crude enzyme from the fermented matter was found to have a profound effect on the enzyme production. Results from Table 1 show that maximum enzyme yield (660 and 470,000 U/g for α-amylase and protease, respectively) was observed when tap water was used for α-amylase and protease extraction compared to other moistening agents.

Conclusion

In this study, it has been indicated that *B. licheniformis* is able to yield both α-amylase and protease by using the SSF method in the presence of RH as substrate. These enzymes will be able to be used in the paper, food, detergent and textile industries due to their economical conditions. In addition, the utilization of this agro-industrial waste, on the one hand, provides alternative substrates, and, on the other, helps in solving pollution problems, which otherwise may hinder its disposal.

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