Production and Optimization Parameters of Amylases from *Bacillus subtilis* RSKK96 Under Solid State Fermentation

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The production of extracellular α-amylase by producing *Bacillus subtilis* RSKK96 was studied under solid state fermentation (SSF). Various agroresidues as substrate were studied for enzyme production. The highest enzyme yield was expressed with cotton stalk substrate (1016.3 ± 32.6 U $g^{-1} \cdot 10^{-3}$). Production parameters were optimized as incubation time 72 hours, incubation temperature 37 °C, agitation speed 150 rpm, inoculum size 35 %, initial moisture content 30 %, initial pH 7.0. Supplementation of the fermentation medium with carbon and metal salt sources decreased the enzyme production. Among different nitrogen sources supplemented, ammonium nitrate (1 %) showed maximum enzyme production (1483.1 ± 32.5 U $g^{-1} \cdot 10^{-3}$). Enzyme activity was observed as 73 % at 100 °C.

Key words:
α-Amylase, *Bacillus subtilis* RSKK96, solid substrate fermentation, cotton stalk

Introduction

Alpha amylases (endo-1,4-α-D-glucan glucanohydrolase, E.C. 3.2.1.1) are extracellular endo enzymes that randomly cleave the 1,4-α linkage between adjacent glucose units in the linear amylose chain and ultimately generate glucose, maltose and maltotriose units.[1] Amylases are among the most important enzymes in present-day biotechnology. The amylase family of enzymes is of great significance due to its wide area of potential application.[2] The extensive application of amylases in the food industry, such as baking, brewing, preparation of digestive aids, production of chocolate cakes, moist cakes, fruit juices, starch syrups, etc., has paved the way for their large scale commercial production.[3–6]

Amylases have been obtained from submerged fermentation (SmF) because of the ease of handling and greater control of environmental factors such as temperature and pH.[1] However, this process is cost intensive due to low concentrations of products and the consequent handling and disposal of a large volume of water during down-stream processing.[7] The cost of enzyme production in submerged fermentation (SmF) is high, which necessitates reducing the production costs through alternative methods. The contents of synthetic media are very expensive and these contents might be replaced with more economically available agricultural by-products for reducing the cost of the medium.[8] The use of agricultural wastes makes solid state fermentation (SSF) an attractive alternative method.[9]

SSF is generally characterized by the growth of microorganism within particles of a solid substrate in the presence of varying amounts of water. The solid substrate acts as a source of carbon, nitrogen, minerals and growth factors, and has a capacity to absorb water necessary for microbial growth. In this process, the solid substrate not only supplies the nutrients to the microbial culture growing in it but also serves as an anchorage for the cells.[10] As the microorganisms in SSF are growing under conditions similar to their natural habitats, they may be able to produce certain enzymes and metabolites more efficiently than in submerged fermentation.[11] SSF has many advantages over SmF, including superior productivity, simple technique, low capital investment, low energy requirement and less water output, better product recovery and lack of foam build up, and is reported to be the most appropriate process for developing countries.[12] A further advantage of SSF is that it employs cheap and easily available substrates, such as agriculture and food industry by-products.[13]

Agro-industrial residues are generally considered the best substrate for SSF processes and enzyme production.[14] A number of such substrates have been employed for the cultivation of microorganisms to produce α-amylase.[15] Cost and availability are important considerations and therefore

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the selection of an appropriate solid substrate plays an important role in the development of efficient SSF processes.[16]

Inexpensive agriculture and agro-industrial residues represent one of the most energy-rich sources on the planet can be used as a substrate in SSF. These residues are in fact, one of the best reservoirs of fixed carbon in nature.[17] The composition concentration of medium components and fermentation conditions greatly affect the growth and production of extracellular enzymes from microorganisms. On preliminary cost analysis clearly suggested, a net savings of about 60 and 50 % on fermentation medium cost and the expenditure on down-stream processing, respectively, as compared to the presently employed SmF technique was evident.[12,18] It is known that SSF is mainly confined to processes involving fungi and is not suitable for bacterial cultures because of higher water activity requirements.[12] However, successful bacterial growth and production α-amylase by using the SSF technique is known in many natural fermentations.[19] The genus *Bacillus* is a major source of some industrial enzymes, and *B. amyloliquefaciens* one of the most widely used species for the bulk production of α-amylase.[20] *Bacillus* species are considered to be the most important sources of α-amylase and have been used for in SSF enzyme production.[2]

In this study, we have evaluated the feasibility of easily available substrates in SSF for the production of α-amylase by *Bacillus subtilis* RSKK96. For this purpose, the effects of incubation temperature, agitation speed, inoculum size, moisture level, initial pH, various carbon and nitrogen sources, and metal salts, and incubation time were investigated. Effect of temperature on enzyme activity was also studied.

Materials and methods

Microorganism

B. subtilis RSKK96 obtained from Refik Saydam Hıfzıssıhha Institute, Ankara, Turkey was used as a source of α-amylase. *B. subtilis* RSKK96 was grown on nutrient agar at 37 °C for 24 h for inoculum preparation. A loopful of the growth was transferred to Laura broth (LB) liquid medium (1 % yeast extract, 0.5 % peptone, 0.5 % NaCI, pH 7.0).

Substrates

Wheat bran (WB), rice husk (RH), lentil husk (LH), cotton stalk (CS), coarse meal of corn (CMC) and coarse meal of millet (CMM) were obtained from Diyarbakır, Turkey. The substrates were ground into a coarse powder with a blender.

Enzyme production in SSF

Solid state fermentation

In an attempt to choose a potential substrate for SSF which supports amylase production, various agroresidues like WB, RH, LH, CS, CMC and CMM were screened individually. SSF was carried out by taking 3 g of dry substrate in a 100 mL Erlenmeyer flask to which distilled water was added to adjust the required moisture level. The contents of the flasks were mixed and autoclaved at 121 °C for 15 minutes. Inoculated flasks were shaken at 150 rpm at 37 °C for 144 h. The contents of the flasks were harvested and assayed every 24 h.

Enzyme extraction

The enzyme from the fermented bacterial bran was extracted twice with tap water. The slurry was squeezed through a damp cheesecloth. Extracts were pooled and centrifuged at 4 °C for 15 minutes at 10000 rpm to separate small particles of different substrates, cells and spores. The brown, clear supernatant was used in enzyme assays.[21] Extraction was done in the following conditions; inoculum size 35 % (by volume per mass), moisture level 30 % (by volume per mass), agitation speed 150 rpm, incubation time 72 h, incubation temperature 37 °C, initial pH 7.0.

Enzyme assay

α-Amylase activity was determined by the procedure of Bernfeld using soluble starch as a substrate.[22] The reaction mixture containing 200 μL of 1 % substrate in 0.1 mol L^{-1} phosphate buffer (pH: 7.0) and 150 μL of enzyme solution was incubated for 30 minutes at 37 °C. The reaction was stopped by adding 400 μL of 3,5-dinitrosalicylic acid solution followed by heating in a boiling water bath for 5 minutes and cooling at room temperature, and then 8 mL of deionized water was added. Absorbance of each solution containing the brown reduction product was measured at 489 nm in a UV-Visible spectrophotometer.

One unit (U) of α-amylase activity was defined as the amount of enzyme that releases 1 μmol of reducing sugar as maltose per minute, under assay conditions and expressed as U g^{-1} of dry substrate.

All the experiments are independent of each other. Results are represented as the average values

of at least three experiments with mean ± S.D. of at least three experiments.

Assay of protein concentration

The protein concentration was determined by the Lowry method by using bovine serum albumin used as a standard.[23]

Effect of process parameters on α-amylase production in SSF

The optimization of medium components and fermentation process is of primary importance in any fermentation process. Combinations of the best substrates were employed for further optimization of process parameters, namely initial moisture content (20, 30, 40, 50, and 60 %), incubation time (24, 48, 72, 96, 120, 144, 168 and 192 h), incubation temperature (30, 37, 40, 45 and 50 °C), initial pH of the medium (pH 4.0–10.0), inoculum size (5–80 %), agitation speed (60, 100, 120, 150, 180, and 200 rpm), while nutrient supplementation such as inorganic nitrogen sources 1 % (by mass) (ammonium nitrate, sodium nitrate, ammonium chloride and ammonium sulphate), organic nitrogen sources (peptone, tryptone, yeast extract, beef extract, urea, and casein), and added metal salts 0.1 % (by mass) $FeSO_4 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$ and $CaCl_2$ were optimised. To study the efficacy of various inducers, the medium was supplemented independently with 1 % mannose, xylose, lactose, sucrose, fructose, galactose, glucose, and arabinose.

Effect of temperature on the enzyme activity

Optimum temperature for enzyme activity was determined by conducting the assay at different temperatures ranging from 25 to 100 °C.

Results and discussion

Screening of agroresidues as substrates for SSF

Solid-state fermentation (SSF) is fermentation of solid substrates at low moisture levels or water activities; however, the substrate must possess enough moisture to support growth and metabolism of the microorganism.[24] In SSF, the selection of a suitable solid substrate for a fermentation process is a critical factor and thus involves the screening of a number of agro-industrial materials for microbial growth and product formation.[25]

Different solid substrates were found to effect the production of enzymes. As it is shown in Table 1, all the substrates supported enzyme formation by the culture, while CS proved superior to the other substrates. A highest titer of α-amylase production (1016.3±32.6 U $g^{-1} \cdot 10^{-3}$) was obtained in a medium containing CS alone as the substrate. The order of substrate suitability was CS>RH>CMC>CMM>LH>WB. In subsequent experiments, therefore, CS was used as the substrate for the production of α-amylase.

The experiment was done in triplicate. Error bars show the percent error.

Optimization of solid-state fermentation

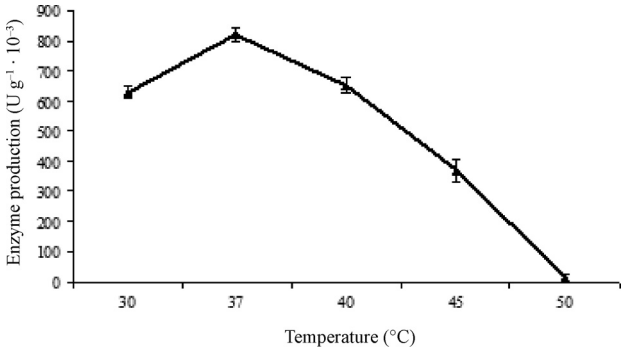
The effect of temperature on enzyme production during fermentation showed that the optimum temperature for maximum yield of amylase was 37 °C for CS with 818.3±21.1 U $g^{-1} \cdot 10^{-3}$ (Fig. 1). Previously, 37 °C were reported as optimum temperature for α-amylase production by several authors.[8,19] Temperature plays a significant role in the development of the biological process as it influences protein denaturation, enzyme inhibition and cell growth.[26]

Enzymes are susceptible to mechanical force, which may disturb the elaborate shape of complex molecule to such a degree that denaturation oc-

Table 1 – *Effect of different substrates on the production of Bacillus subtilis RSKK96 α-amylase by solid state fermentation*

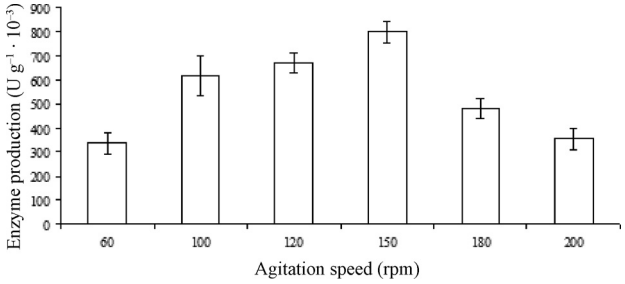
	Substrates					
Hour	CS	RH	CMM	LH	WB	CMC
	Enzyme production (U $g^{-1} \cdot 10^{-3}$)					
24	712.1±23.0	647.0±15.9	391.6±72.3	292.8±47.4	186.2±21.3	396.7±13.8
48	912.1±91.4	672.9±30.4	566.7±39.9	320.2±16.7	121.6±19.8	419.4±69.4
72	1016.3±32.6	905.9±43.9	371.7±73.1	322.7±22.3	910.9±44.6	796.7±16.2
96	973.5±46.4	744.9±19.4	354.0±21.4	355.5±42.7	116.4±22.0	719.4±47.3
120	831.7±89.7	685.6±29.1	311.7±8.7	681.1±32.8	93.2±24.8	332.3±34.0
144	665.6±35.6	613.1±31.0	272.3±43.9	736.9±52.2	96.3±22.5	308.9±40.9

The experiment was done in triplicate. Error bars shoe the percent error.



F i g . 1 – *Effect of temperature on the production of Bacillus subtilis RSKK96 α-amylase (incubation time 72 h, inoculum size 35 % (by volume per mass), moisture level 30 % (by volume per mass), initial pH 7.0, agitation speed 150 rpm). The experiment was done in triplicate. Error bars show the percent error.*

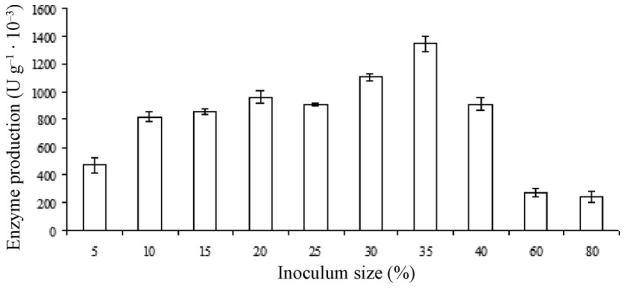
curs.[12] α-Amylase production was investigated at six different speeds (60, 100, 120, 150, 180 and 200 rpm). The optimal agitation speed for maximum α-amylase production (799.1±43.5 U g^{-1} · 10^{-3}) was obtained at 150 rpm (Fig. 2). Similar results were reported by Anto *et al*., 2006 and Tanyıldızı *et al*., 2007.[8,12]



F i g . 2 – *Effect of agitation speed on the production of Bacillus subtilis RSKK96 α-amylase (incubation time 72 h, incubation temperature 37 °C, inoculum size 35 % (by volume per mass), moisture level 30 % (by volume per mass), initial pH 7.0). The experiment was done in triplicate. Error bars show the percent error.*

The inoculum level was also an important factor for the production of α-amylase. The highest enzyme production (1341.7±52.3 U g^{-1} · 10^{-3}) was obtained at an inoculum level of 35 % (v/w). A higher inoculum size may increase moisture content and lead to a decrease in growth and enzyme production; this may be due to the limiting nutrients at higher inoculum size and a lower inoculum size may require a longer time for fermentation to form the desired product.[1,19,27] The results from this study indicate that 35 % inoculum size was optimal, balancing enzyme and biomass production (Fig. 3).

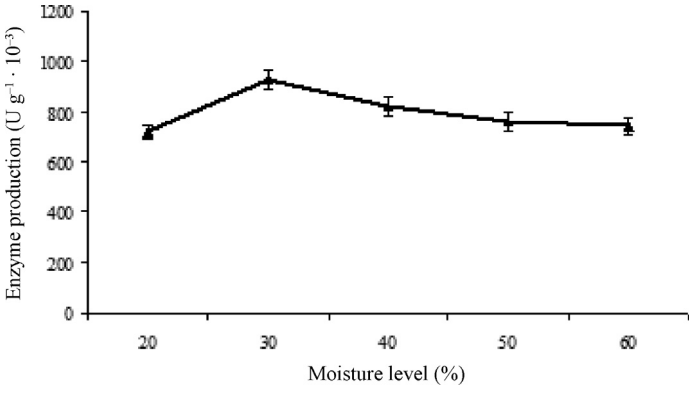
Initial moisture content of the substrates is known to critically influence bacteria growth and enzyme production in SSF. The optimal moisture



F i g . 3 – *Effect of inoculum size on the production of Bacillus subtilis RSKK96 α-amylase (incubation time 72 h, incubation temperature 37 °C, moisture level 30 % (by volume per mass), initial pH 7.0, agitation speed 150 rpm). The experiment was done in triplicate. Error*

level of substrate for enzyme production was found to be 30 %, with 926.9±36.6 U g^{-1} · 10^{-3} when compared to 20, 40, 50 and 60 % of CS (Fig. 4). The optimum moisture content for growth and substrate utilization depends on the organisms and the substrate used for cultivation. For example, Balkan and Ertan (2007) reported that initial moisture content (30–80 %) was a critical factor for solid-state fermentation processes because this variable influences growth, biosynthesis and secretion of different metabolites.[28] A reduction in enzyme production at high initial moisture content may be due to a reduction in substrate porosity, changes in the structure of substrate particles and reduction of gas volume.[15,19] In addition, reduction in enzyme production may result from lower bacterial growth.[29] This may be due to the use of different species of bacteria.

Among the physicochemical parameters, the pH of the growth medium plays an important role by inducing morphological changes in the organism and in enzyme secretion.[1] The production of α-amylase is very sensitive to initial pH of the fermenta-



F i g . 4 – *Effect of moisture level on the production of Bacillus subtilis RSKK96 α-amylase (incubation time 72 h, incubation temperature 37 °C, inoculum size 35 %, initial pH 7.0, agitation speed 150 rpm). The experiment was done in triplicate. Error bars show the percent error.*

tion medium.[30] Fig. 6 depicts that pH played a sensitive role in enzyme production and growth of *B. subtilis* RSKK96. The enzyme production was maximum when initial medium pH was 7.0, which yielded 923.3±56.4 U $g^{-1} \cdot 10^{-3}$ (Fig. 5). Further increase in pH resulted in decrease of α-amylase production by the *B. subtilis* RSSK96. Haq *et al*. (2003) reported pH = 7.5–8.0 was the best for the production of alpha amylase by *Bacillus subtilis*.[31] Variations in pH result from the substrate consumption (*e.g.* protein hydrolysis) and/or metabolite production (*e.g.* organic acids). Generally, agro-industrial wastes possess unique buffering action and have advantages for enzyme production. Therefore, in the subsequent experiments, the initial pH of the fermentation medium was adjusted to 7.0.

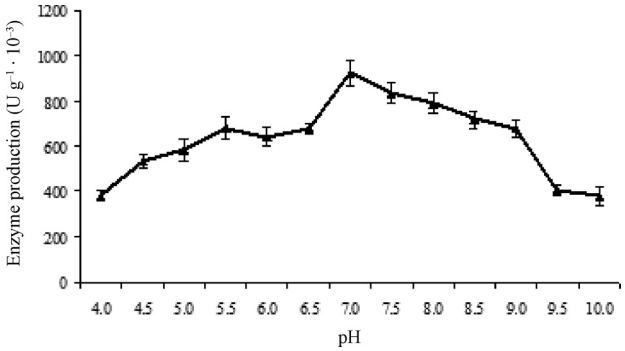


Fig. 5 – *Effect of initial pH on the production of Bacillus subtilis RSKK96 α-amylase (incubation time 72 h, incubation temperature 37 °C, inoculum size 35 %, moisture level 30 % (by volume per mass), agitation speed 150 rpm). The experiment was done in triplicate. Error bars show the percent error.*

The supplementation of CS with different carbon sources such as, mannose, xylose, lactose, sucrose, fructose, galactose, glucose, and arabinose at 1 % concentration in the production of α-amylase by *B. subtilis* RSKK96 was investigated in order to obtain a suitable medium for α-amylase production. As shown in Table 2, in comparison with the control (1142.8±40.9 U $g^{-1} \cdot 10^{-3}$), there was no significant increase in enzyme yield in the case of supplementation of carbon sources. The production of α-amylase by *B. subtilis* RSKK96 was greatly suppressed when the bacterium was grown on readily metabolizable sugars, since a low basal activity of α-amylase was detected in the culture medium in the presence of mannose, xylose, lactose, sucrose, fructose, glucose, and arabinose. A similar result obtained that carbon sources such as glucose, maltose and did not enhance α-amylase production by *B. coagulans* in solid-state fermentation using wheat bran.[21] Galactose had no impact on enzyme production while the other carbon sources exhibited a repressive effect. Easily metabolizable carbohydrates may result in the better growth of the bacteria along with reduction in the enzyme formation.[32]

Table 2 – *Effect of carbon sources on the production of Bacillus subtilis RSKK96 α-amylase (incubation time 72 h, incubation temperature 37 °C, inoculum size 35 %, moisture level 30 % (by volume per mass), inital pH 7.0, agitation speed 150 rpm)*

Carbon source (1 %)	Enzyme production (U $g^{-1} \cdot 10^{-3}$)
*Control	1142.8±40.9
Mannose	458.6±36.8
Arabinose	979.2±79.8
Sucrose	973.0±34.6
Glucose	597.6±39.0
Galactose	1128.2±37.5
Fructose	802.2±37.3
Lactose	877.7±25.7
Xylose	413.3±23.4

*Control contains only rice bran and tap water.
The experiment was done in triplicate. Error bars show the percent error.

Addition of organic nitrogen sources such as casein, peptone, tryptone, yeast extract, beef extract, urea, and casein, and inorganic nitrogen source such as ammonium nitrate, sodium nitrate, ammonium chloride and ammonium sulphate to the medium was also investigated. In our studies, as shown in Table 3, in comparison with the control (1185.7±59.1 U $g^{-1} \cdot 10^{-3}$), there was a significant increase in enzyme production in the case of supplementation of ammonium nitrate (1483.1±32.5 U $g^{-1} \cdot 10^{-3}$) which proved to be the best among all the nitrogen sources. Pedersen and Nielsen (2000) and Ramanchandra *et al*. (2004) also reported that nitrate was inferior to ammonia in α-amylase production.[30,6] Urea had no impact on enzyme production while the other nitrogen sources exhibited a repressive effect. Supplementation of additional nitrogen sources in general has been reported to be inhibitory for α-amylase production by microorganisms.[5,31] Ammonium nitrate had been found to be suitable with CS when used in SSF. Apart from a good carbon source, cotton stalk also serves as a nitrogen source, thus an increase in the complex nitrogen source adversely influenced the production of alpha amylase. Since cotton stalk was used, nitrogen requirement could be met from CS.

Addition of metal salts source such as $FeSO_4 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$, and $CaCl_2$ to the medium were investigated. In comparison with the control (1185.7±30.8 U $g^{-1} \cdot 10^{-3}$), the production of α-amylase by *B. subtilis* RSKK96 was suppressed when the bacterium was grown on

Table 3 – *Effect of nitrogen sources on the production of Bacillus subtilis RSKK96 α-amylase (incubation time 72 h, incubation temperature 37 °C, inoculum size 35 %, moisture level 30 % (by volume per mass), inital pH 7.0, agitation speed 150 rpm)*

Nitrogen source (1 %)	Enzyme production ($U\ g^{-1} \cdot 10^{-3}$)
*Control	1185.7±59.1
Sodium nitrate	852.9±45.2
Ammonium sulphate	846.9±37.8
Ammonium nitrate	1483.1±32.5
Ammonium chloride	1043.6±61.9
Beef extract	886.1±64.3
Tryptone	850.5±56.7
Peptone	1012.3±40.8
Yeast extract	685.1±39.2
Urea	1172.7±35.9
Casein	633.6±38.6

*Control contains only rice bran and tap water.
The experiment was done in triplicate. Error bars show the percent error.

CS medium supplemented with metal salts (Table 4). Although there are many reports indicating an enhancement of α-amylase production by salts[33,34] the salt requirement for production of this particular enzyme was apparently provided by the nature of CS. These are important in terms of the cost of enzyme production.

The incubation time for achieving the maximum enzyme level is governed by the characteristics of the culture and is based on growth rate and enzyme production. The *B. subtilis* RSKK96 strain produced high titers of enzyme

Table 4 – *Effect of metal salts sources on the production of Bacillus subtilis RSKK96 α-amylase (incubation time 72 h, incubation temperature 37 °C, inoculum size 35 %, moisture level 30 % (by volume per mass), inital pH 7.0, agitation speed 150 rpm)*

Metal salts source (0.1 %)	Enzyme production ($U\ g^{-1} \cdot 10^{-3}$)
*Control	1185.7±30.8
$MgSO_4$	949.4±46.6
$ZnSO_4$	776.4±41.3
$CaCl_2$	1053.0±61.2
$CuSO_4$	1041.3±36.9
$FeSO_4$	1087.6±29.8

*Control contains only rice bran and tap water.
The experiment was done in triplicate. Error bars show the percent error.

(1403.7±20.7 U U $g^{-1} \cdot 10^{-3}$) at 72 h of incubation (Fig. 6). The production of enzyme decreased after 72 h for CS. A similar result was reported by Gangadharan *et al*. 2006.[1] Enzyme production is related to the growth of the microorganism. Growth of the organisms would have reached a stage (due to insufficient nutrients) that indirectly stimulated production of secondary metabolites.[35]

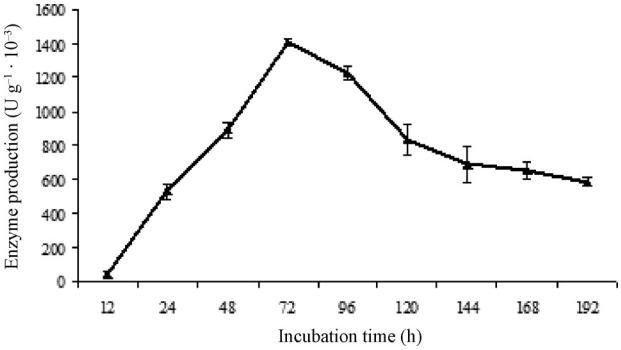


Fig. 6 – *Effect of incubation time on the production of Bacillus subtilis RSKK96 α-amylase (incubation temperature 37 °C, inoculum size 35 % (by volume per mass), moisture level 30 % (by volume per mass), initial pH 7.0, agitation speed 150 rpm). The experiment was done in triplicate. Error bars show the percent error.*

α-Amylase produced by *B. subtilis* RSKK96 showed considerable enzyme activity in the ranges from lower to higher temperature (Fig. 7). At 100 °C, 73 % activity was observed compared to the optimum enzyme activity at 60 °C. As starch liquefaction is generally carried out at higher temperatures of 70–90 °C, the thermostable α-amylases are of great significance.[36] Similar results were obtained by other studies.[8,36] On the contrary, the highest α-amylase activity occurred around 135 °C and the enzyme retained 70 % of its maximum activity up to 165 °C, as reported earlier.[37] Thus, further studies on the thermal stability of α-amylase enzyme produced by *B. subtilis* RSKK96 have to be carried out to confirm its applications for starch liquefaction.

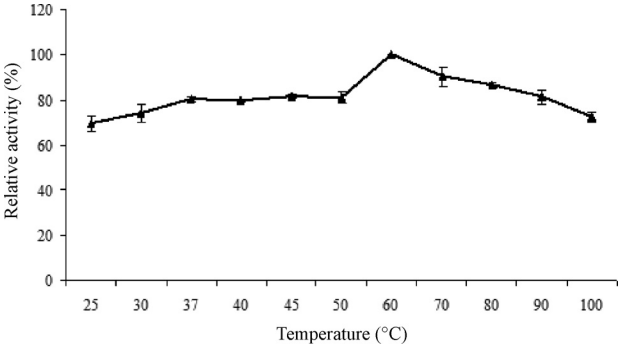


Fig. 7 – *Effect of temperature on optimum activity of Bacillus subtilis RSKK96 α-amylase. The experiment was done in triplicate. Error bars show the percent error.*

Conclusions

Commercial α-amylase is usually produced by submerged fermentation; however, SSF appears promising due to the natural potential and advantages it offers. Based on the present study, it appears that cotton stalk, which is inexpensive and readily available agricultural substance, could replace the commercial and more expensive substances in the development of a suitable economic fermentation medium for obtaining high yields of α-amylase. However, the present study was entirely a laboratory-scale study, and it has to be further improved for a large-scale SSF.

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