

Production of α -amylase by thermotolerant *Bacillus subtilis* in the presence of some carbon, nitrogen containing compounds and surfactants

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Abstract - The effect of some carbon, nitrogen sources and surfactants on the α -amylase production of *Bacillus subtilis* isolated from hot-spring water was investigated. Galactose, glucose, xylose, sucrose, fructose or corn starch were used as a carbon sources for production of α -amylase. Methionine, glycine, aspartic acid, lysine, $(\text{NH}_4)_2\text{SO}_4$, NH_4Cl , $(\text{NH}_4)_2\text{S}_2\text{O}_8$ and NH_4NO_3 were used as a organic and inorganic nitrogen sources, respectively. The higher α -amylase production was determined in corn starch, sucrose, fructose and galactose compared to other sugars and with lysine and methionine than compared with other organic nitrogen sources. Nitrate and aspartic acid are clearly not as good a nitrogen source for α -amylase production. When Triton X100, sodium dodecyl sulphate (SDS) and Tween 40 were employed in growth medium, although bacterial growth was found high, enzyme production was not detectable. Soluble starch and yeast extract were used in order to evaluate the influence of the medium composition on the α -amylase production instead of sole carbon and nitrogen sources. Maximum enzyme production was found in 3.5 % and 1.5% soluble starch and yeast extract growth media, respectively.

Key words: *Bacillus subtilis*, α -amylase, carbon and nitrogen sources.

INTRODUCTION

Extracellular enzymes produced by *Bacillus* spp. are used in many industrial application (Hiller et al., 1996). The starch processing industry is unique within the industrial enzyme sector, in that the use of thermostable enzyme is essential for the industry. Especially α -amylases are one of the industrially important enzymes produced in higher quantities and represents ~12% of the sales value of the world market for enzymes. In addition to its use in starch processing industries, the ability of α -amylase to cause mid chain random degradation of starch has been of vital importance to several other industries. Among these are the brewing, textile, paper, foods and detergent industries. The process of amylase biosynthesis has usually been investigated under experimental conditions that include high aeration, an effi-

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ciently metabolizable carbon source, a complex nitrogen source and an optimal temperature (Lonsane and Ramesh, 1990). There are different reports on the influence of various carbon and nitrogen sources on α -amylase production (Pedersen and Nielsen, 2000; Carlsen and Nielsen, 2001).

In the present work, the influence of different carbon, nitrogen sources, surfactants, soluble starch and yeast extract on α -amylase production in *Bacillus subtilis* was examined.

MATERIALS AND METHODS

Microbial strain. A Gram-positive, spore forming thermotolerant *B. subtilis* was used as biological material isolated from Çermik Belkishatun hot-spring water, in Diyarbakır, Turkey (Aytekin *et al.*, 1994).

Inoculum medium. Soluble starch 2%, peptone 0.5%, yeast extract 0.25%, K_2HPO_4 0.2%, $MgSO_4 \cdot 7H_2O$ 0.1% (w/v), pH 7.

Soluble starch-Beef extract (SB) medium. Soluble starch 2%, beef extract 1%, yeast extract 0.2%, $CaCl_2$ 0.02%, $MgSO_4 \cdot 7H_2O$ 0.01% (w/v), pH 7 (Babu and Satyanarayana, 1993).

Enzyme activity. α -Amylase activity was determined by procedure of Bernfeld (1955) using soluble starch as a substrate. The reaction mixture containing 200 μ L soluble starch (1% w/v) in 0.1 M phosphate buffer (pH 7) and 150 μ L enzyme solution was incubated for 30 min at 37 °C. The reaction was stopped by adding 400 μ L 3,5-dinitrosalicylic acid solution, followed by heating in a boiling water bath for 5 min, 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 by UV-Visible Spectrophotometer.

Determination of α -amylase secretion time. *Bacillus subtilis* taken into inoculum medium was grown in SB medium at various inoculum concentrations (0.5%; 1%; 1.5%, 2%) for 48 h at 37 °C at a shake rate of 200 rev min⁻¹ and samples were taken at definite intervals. Cell density and amylase activity were measured to determine appropriate inoculum concentration and the best secretion time.

Effect of some different carbon, nitrogen sources and surfactants on α -amylase production. *Bacillus subtilis* taken into inoculum medium was grown in SB medium supplemented with galactose, glucose, xylose, sucrose, corn starch or fructose instead of 2% soluble starch or 0.2% yeast extract (w/v). Some organic and inorganic nitrogen sources such as methionine, glycine, aspartic acid, lysine, $(NH_4)_2SO_4$, NH_4Cl , $(NH_4)_2S_2O_8$ and NH_4NO_3 were also added to SB medium instead of 1% beef extract and 0.2% yeast extract (w/v) (Babu and Satyanarayana, 1993). Triton X100, sodiumdodecyl sulphate (SDS) or Tween 40 were added in different SB media for determining the effect of these surfactants on enzyme production.

TABLE 1 – Effect of different nitrogen sources (1%) on α -amylase production

Nitrogen source	Cell density (absorbance A_{600})	α -Amylase production U/L (x1000)
Control	1.893	23.20
Methionine	0.250	31.84
Glycine	0.233	24.96
Aspartic acid	0.254	0
Lysine	0.567	37.92
$(\text{NH}_4)_2\text{SO}_4$	0.270	11.68
NH_4Cl	0.202	22.40
$(\text{NH}_4)_2\text{S}_2\text{O}_8$	0.187	16.24
NH_4NO_3	0.309	6.8

Effect of soluble starch and yeast extract on α -amylase production. Bacteria were grown in two different SB media containing 0.25-3.5% soluble starch, 0.02% CaCl_2 and 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.25-3.5% yeast extract, 0.02% CaCl_2 and 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (w/v).

Enzyme activity was measured in supernatant at all conditions.

RESULTS AND DISCUSSION

Inoculum concentration in growth medium is important in enzyme studies. It was shown that high inoculum concentration inhibits amylase production when inoculated from preincubation to second growth medium (Haddad *et al.*, 1974). With this aim, appropriate inoculum concentration was found to be 2% and incubation time 19 h.

It has been found that bacteria increase exoenzyme synthesis using protein, peptide and peptone in growth medium. Moreover, organic and complex nitrogen sources increase amylase production while inorganic nitrogen sources decrease it. (Babu and Satyanarayana 1993; Pedersen and Nielsen, 2000).

Among aminoacids tested, lysine and methionine supported maximum enzyme production followed by glycine (Table 1). The activity was not observed with aspartic acid. Among various inorganic nitrogen sources tested, NH_4Cl and $(\text{NH}_4)_2\text{S}_2\text{O}_8$ favoured enzyme secretion while $(\text{NH}_4)_2\text{SO}_4$ and NH_4NO_3 caused no enzyme secretion (Table 1).

This result may be explained that bacteria increases its exoenzyme synthesis by using organic nitrogen compounds during enzyme synthesis.

*All media were autoclaved at 121 oC and 15 psi for 20 min. The amino acids, sugars and inorganic compounds added to the media after cooling, were autoclaved separately.

TABLE 2 – Effect of different carbon sources (2%) on α -amylase production

Carbon source	Cell density (absorbance A_{600})	α -Amylase activity U/L ($\times 1000$)
Control	1.893	23.20
Galactose	1.606	17.60
Glucose	1.850	13.28
Xylose	1.052	0
Sucrose	2.401	21.44
Fructose	2.352	15.52
Corn starch	1.220	32.16

Several previous attempts have been made to observe the effect of carbon sources on α -amylase production by *Bacillus* spp. (Chandra *et al.*, 1980; Wind *et al.*, 1994). In our study the activity was higher in the presence of corn starch, sucrose and galactose than glucose and fructose but there was no trace of enzyme in xylose growth medium, respectively (Table 2). We can explain this result by the repression of α -amylase synthesis due to the repression of low mass metabolizable sugars in enzyme biosynthesis (Saito and Yamamoto, 1975). It is likely that sucrose and corn starch thorough hydrogen bonds and hydrophilic interactions with proteins.

No correlation was found between cell density and enzyme production. The reason may be due to the fact that growth and enzyme synthesis are controlled by different regulatory mechanisms.

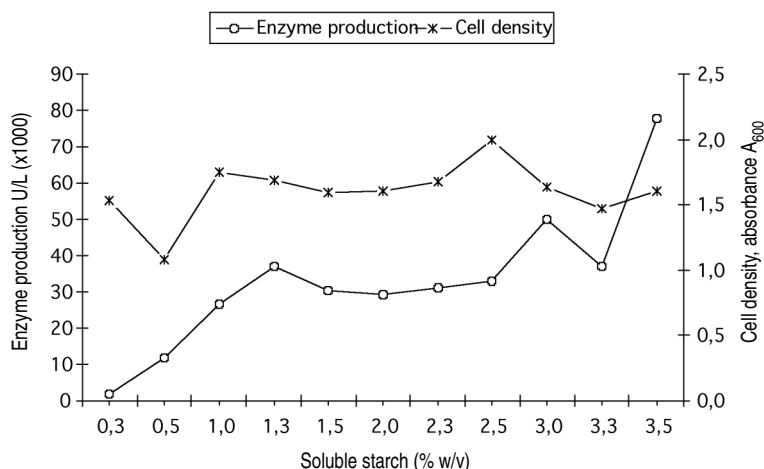


FIG. 1 – Growth and α - amylase production (U/L) at different soluble starch concentrations.

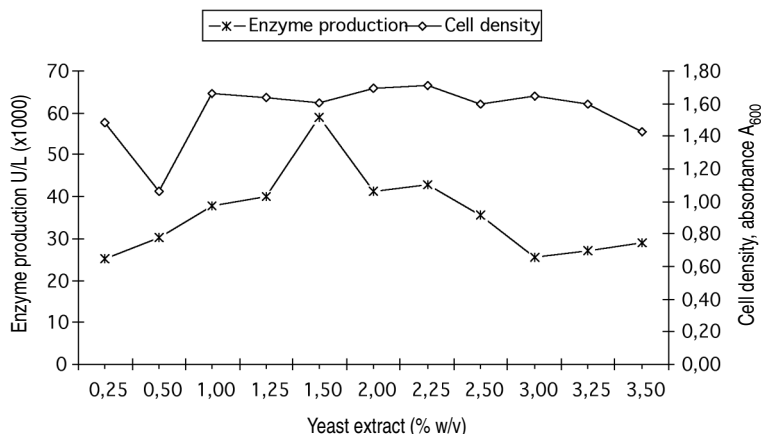


FIG. 2 – Growth and α - amylase production (U/L) at different yeast extract concentrations.

Enzyme production in culture supernatants of surfactant-containing such as Triton X 100, SDS and Tween 40 was not detectable while growth was accelerated, 1.442, 2.472 and 2.281 cell density (A_{600}), respectively. Probably, the initially applied surfactants still be present, thus influencing the structure of proteins and therefore the activity of enzyme. This may decrease both enzyme activity and also secretion. In addition, the effects of the membrane and on the protein export mechanisms may contribute to a decreased α -amylase production.

Yeast extract and starch were investigated since they are used for industrial protein production. Bacterial growth was found almost same when different starch and yeast extract concentrations were added to the growth media at all conditions. Enzyme production was higher at high starch concentrations than in the yeast extract growth medium (Fig. 1 and 2). These results indicate that treatment with starch or complex nitrogen sources such as yeast extract increase the bacterial growth and α -amylase production. Starch and yeast extract have been reported to be an inducer for amylase synthesis by some workers (Burbidge and Collier, 1968; Pedersen and Nielsen, 2000).

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