The Effects of Different Grain Brans Used as Substrates on Resistance to Catabolite Repression in Solid-State Fermentation Process

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Summary: Production of α-amylase from *Penicillium brevicompactum* was investigated in solid-state fermentation (SSF) using as substrate wheat bran (WB), rye bran (RB) and barley bran (BB) enriched with different amount of glucose or not. Consumption of glucose by fungal cells in WB and RB cultures was more effective than BB cultures. Optimal moisture levels for maximal α-amylase production in WB, RB and BB cultures without glucose were 55, 65 and 35 %, respectively. Water absorption capacities of substrates were WB>RB>BB. In SSF process, decrease in enzyme production was greater in high moisture level than optimal moisture level. According to the other two cultures, production of α-amylase from *P. brevicompactum* was strongly inhibited in higher moisture levels than optimal moisture levels in BB cultures enriched with 500 mg/g glucose.

Key words: *Penicillium brevicompactum,* Glucose, Catabolite repression, α-amylase, SSF.

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 amylase chain and ultimately generate glucose, maltose, and maltotriose units [1].

Fungal and bacterial α-amylases such as most other types of industrial enzymes can be obtained either by submerged fermentation (SmF) or solid state fermentation (SSF). Most enzyme manufacturers produce enzymes using SmF techniques [2]. SSF is an alternative culture method that has gained researchers attention over the past 20 years [3]. There is a significant interest in using SSF techniques to produce a wide variety of enzymes, mainly from mold origin, as indicated by the growing number of research papers in the literature and the marketing [2]. SSF are fermentations 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. The water content of a typical SmF is more than 95 %. The water content of solid mash in SSF often varies between 40 % and 80 % [4].

Inducers are the chemical signal that is required for the microbial synthesis of many enzymes. The inhibition of this synthesis is called catabolite repression (CR) and the abundance of glucose, glycerol or other readily found fermentable carbon sources inhibit this enzyme synthesis [5]. CR of enzyme synthesis prevents the use of high glucose medium normally used to prevent sporulation during fermentation [6]. A strong CR is caused by SmF using microorganisms when they are in the presence of glucose, fructose or other highy metabolizable carbon sources [7]. In the SmF, glucose and readily metabolizable monosaccharides inhibit α-amylase synthesis at transcription level [8]. CR caused by glucose and other easily metabolizable sugars in the production of amylase by microorganisms developed in SmF is well documented [9-12].

Agro industrial residues are generally considered the best substrates for the SSF processes and enzyme production in SSF [13]. The utilization of by-products and waste from food and industrial sources has several advantages over SmF, such as superior productivity, simpler techniques, reduced energy requirements, improved product recovery and reduced production costs since they supply the microorganism with some nutritive substances [14]. On the other hand, the ability of SSF to minimize CR has been described for the production of different hydrolytic enzymes [15].

Gonzales and Torres [16] expressed that resistance to CR in SSF system is a relative characteristic depending on the nature of substrate, and α-amylase produced in this system are resistant to CR. Moreover, within our knowledge, resistance to CR in SSF systems where wheat bran (WB) was used as substrates enriched with glucose was reported in the previous α-amylase production studies [9, 15, 17-19]. Due to this fact, in addition to WB, rye bran (RB) and barley bran (BB) used as substrates in SSF, whether have the ability to minimize the CR caused by glucose in α-amylase production from *Penicillium brevicompactum* was investigated.

Results and Discussion

In the present study, the effects of using WB, RB and BB as substrates on resistance to CR in SSF systems were investigated. On the 6th day of cultivation, at the 65 % moisture level, the production of α-amylase from *P.brevicompactum* in WB, RB and BB cultures containing different concentrations (50, 250, 500, 1250 mg/g) of initial glucose was compared with control medium, without glucose. Relative activities (%) for WB, RB and BB cultures were 100, 90.4, 89.4, 88.4; 93, 87, 86.6, 78; 90, 72, 48, 28.6, respectively (Fig. 1). Relative activities were higher in WB and RB cultures containing different concentrations of glucose comparing to BB. The lost activities were however higher in increasing glucose amounts in BB cultures comparing with other cultures. In BB cultures particularly in 500 and 1250 mg/g glucose concentrations, the loss of activities were 52, 71.4 %, respectively (Fig. 2). Yet, remaining glucose in WB and RB cultures (mg/g) was higher than BB cultures. The remaining glucose concentrations (mg/g) for WB, RB and BB cultures were 0, 21.5, 124, 264.6; 1.36, 27.1, 128.8, 293.3; 28.4, 117, 251, 378.5, respectively (Fig. 3). The more the remaining glucose, the more there were the loss of activity. In BB cultures with enrichment 500 mg/g glucose, the remaining glucose was about 2 times higher and the reduction in enzyme production was 5 times greater than the other cultures. According to these results, SSF process using BB was not resistant to CR but WB and RB cultures were. In addition, consumption of glucose by fungal cells in WB and RB cultures was more effective than BB cultures.

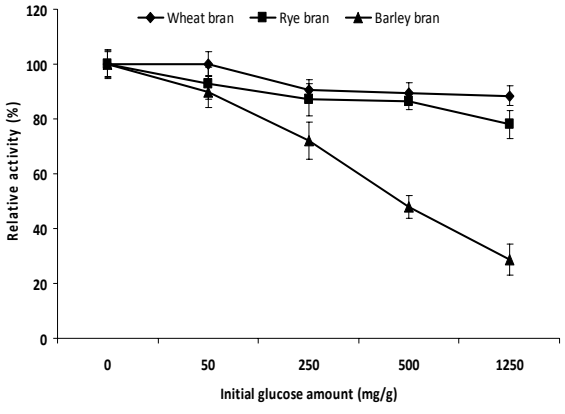

Fig. 1: Effect of initial glucose on the production of α-amylase from *P. brevicompactum* in SSF. Incubation period 6 days, inoculum level 1.5 mL, initial moisture level 65 % (w/v), incubation temperature 30°C, moistening agent acetate buffer (pH 5.0).

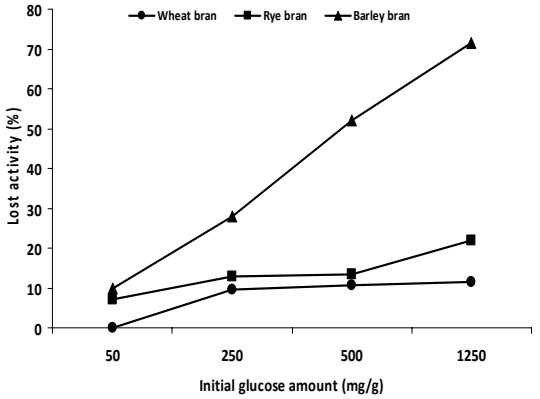

Fig. 2: Lost activity of α-amylase production by *P. brevicompactum* in SSF. For the enzyme assay, reaction mixture consisting of 0.1 mL of enzyme and 0.2 mL of soluble starch (1 %) were incubated at 30 °C for 5 min.

Fig. 3: Residual glucose amounts in SSF systems. For the remaining glucose concentrations, to 0.5 mL of the filtrate in test tubes, add 4.5 mL of *o*-toluidine reagent. Place test tubes in boiling water for 10 min, cool with cold water; add 5 mL of glacial acetic acid. The absorbance was measured at 630 nm.

Initial moisture level in SSF processes is one of the most significant parameters in enzyme production. In SSF processes, substrate must have enough moisture for the metabolism and activity of the microorganism. The absorbent capacities of the each substrate are different. The water which isn't absorbed by substrate will increase diffusion in cultures. In conclusion, the unabsorbed water will accelerate the reach of glucose to fungus or slow it down and physiology of fungus will be affected. The enzyme production was examined in WB, RB and BB cultures with and without enrichment 500 mg/g glucose at different moisture levels (25, 35, 45, 55,

65, 75, and 85 %). The maximum enzyme production (647.14 U/gds) in BB control mediums was determined at 35 % of initial moisture (Fig. 4). At 25 % moisture level, in BB cultures, for with and without enrichment 500 mg/g glucose, the enzyme productions (U/gds) were 621.4 and 615.6, respectively (Fig. 4). At the other moisture levels, however, the enzyme productions were lower than control mediums. In WB and RB control mediums, the maximum enzyme productions (which were 720 and 167.5 U/gds, respectively) were found at 55 and 65 % moisture levels, respectively (Fig. 5, 6). The reduction in enzyme production in WB cultures was higher in the moisture levels which were higher than 55 %. In RB cultures, however, there was a decline in production when the moisture levels were higher than 65%. When we compare BB, WB and RB cultures, which were at 85 % moisture level, to the control mediums, the decrease in enzyme production were 3.6, 1.3 and 1.3 times, respectively.

These results are similar with the opinion of Viniegra-Gonzales and Favela-Torres [16] that suggests "Resistance to CR in SSF cultures is a relative aspect that may change with respect to nature of support". SSF is a term that describes the process where insoluble materials in water are used for microbial growth. The amount of water added in the fermentative processes of SSF should not be more than the capacity of the solid bed in which the microorganisms grow. In SSF water is necessary where it is present in thin layers and occasionally, absorbed inside the substrate [20]. At the presence of glucose, the reason enzyme production is different in WB, RB and BB cultures may be due to substrates different absorbent features because all the other parameters tested and the fungi are the same. As it is seen Fig. 4, 5 and 6, optimal moisture levels of substrates were different for WB, RB and BB cultures without glucose is as follows; 55, 65 and 35 %, respectively. These results showed that water holding capacities of the substrates are different and water activity of substrates has a strong influence on microbial activity [21]. Filamentous fungi form new branches as well as tubular hyphae which, elongates at the tips, after germination. Their morphology helps in the colonization of the surface substrate matrix in search of nutrients. The metabolites and the enzymes are secreted from the microbial biomass inside the substrate matrix and on the substrate surface . This biomass also consumes the nutrients that are liberated [3]. The reduction in enzyme production was more in high moisture level than optimal moisture level when it is compared to the cultures without glucose.

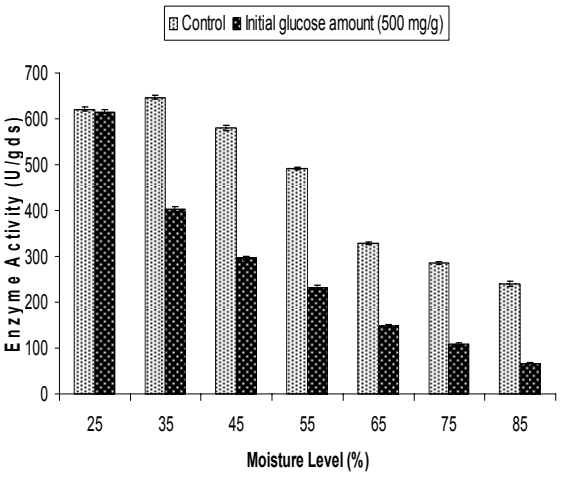

Fig. 4: The effect of moisture level in α-amylase production in SSF processes, where barley bran was used as substrate, with and without enrichment with 500 mg/g glucose. Incubation period 6 days, inoculum level 1.5 mL, incubation temperature 30°C, moistening agent acetate buffer (pH 5.0).

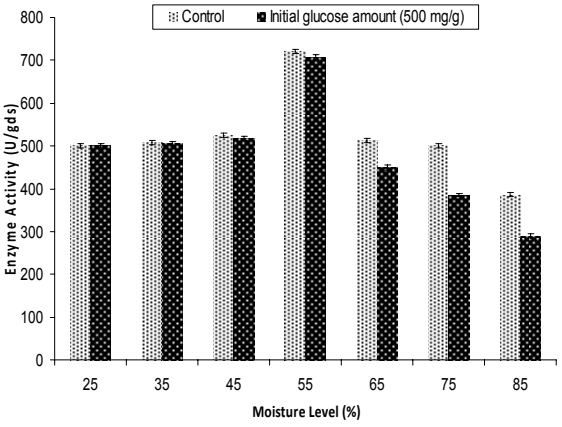

Fig. 5: The effect of moisture level in α-amylase production in SSF processes, where wheat bran was used as substrate, with and without enrichment with 500 mg/g glucose. Incubation period 6 days, inoculum level 1.5 mL, incubation temperature 30°C, moistening agent acetate buffer (pH 5.0).

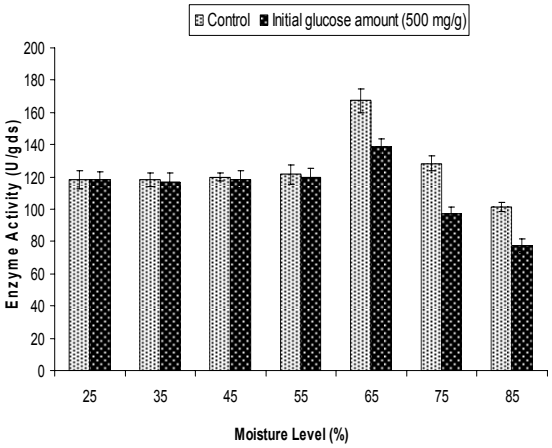

Fig. 6: The effect of moisture level in α-amylase production in SSF processes, where rye bran was used as substrate, with and without enrichment with 500 mg/g glucose. Incubation period 6 days, inoculum level 1.5 mL, incubation temperature 30°C, moistening agent acetate buffer (pH 5.0).

If the saturation capacity of absorbent substrate is exceeded, non-absorbed water will increase diffusion in the SSF process and the glucose will reach fungus easier and affect its physiology, caused morphological changes of fungi. As a result, gradient in the concentration of glucose may affect enzyme production. In SSF processes where WB is used as substrate, it was reported that either CR in α-amylase production from different bacteria and fungi does not occur or is negligible. The α-amylase production by *Bacillus licheniformis* m27 in a SSF system was 19.550 U/mL in the extract even when the medium contained 15 % glucose [17, 18]. The repression was negligible, even when the glucose level was raised to 150 mg/g wheat bran, for both alpha and amyloglucosidase synthesis by *Aspergillus niger* CFTRI 1105 [9].

Experimental

Fungus

P. brevicompactum was isolated from air in Edirne city (Turkey). Morphological, physiological and biochemical characterization of fungus was performed by Aydoğdu H. and Asan A. [22]. It was found to be a good α-amylase producer and its enzyme property was investigated in our previous study [23]. It was maintained on potato dextrose agar slants at 4 °C.

Substrates

Different agricultural by-products such as WB, RB and BB, were used as substrate. They were obtained from the local flour mill (Yayla Flour Mill, Kırklareli, Turkey). The chemical compositions (%) of substrates are as follows;

1. WB; protein, 14.8; fat, 3.9; starch, 20.8; ash, 4.06; dietary fibre, 33.4.
2. RB; protein, 7.6; fat, 4.8; starch, 25.7; ash, 6.1; dietary fibre, 37.5.
3. BB; protein, 11.9; fat, 4.2; starch, 6.9; ash, 6.01; dietary fibre, 22.6.

Inoculum preparation

A volume of 7 mL of sterile distilled water was transferred to a sporulated (7days old) PDA slant culture. The spores were dislodged using the inoculation needle under aseptic conditions and the suspension, with appropriate dilution, was used as inoculum. A volume of 1 mL of spore suspension contained about 1×10^6 spores.

Solid-State Fermentation

The SSF process was carried out in 250 mL Erlenmeyer flasks containing 5 g of substrates (WB, RB and BB), with or without glucose, at different concentrations (50, 250, 500, 1250 mg/g). Acetate buffer (pH 5.0) was used to adjust the moisture content from 25 to 85%. After autoclaving at 121 °C, 20 min., flasks were cooled and inoculated with 1.5 mL inoculum level. After incubation at 30 °C for 6 days, 50 mL 0.1 M acetate buffer (pH 5.0) was added to the fermented Erlenmeyer flasks. The mixture was shaken for an hour (200rpm/min). The slurry was squeezed through muslin cloth. The extract was filtered through Whatman No. 1 filter paper and the filtrate was used as the crude enzyme and to determine remaining glucose concentrations.

Enzyme Assay and Remaining Glucose Determination

Soluble starch (1%) was dissolved in boiling 0.1 M acetate buffer pH 5.0 and then cooled to 30°C. Fresh iodine reagent was prepared by diluting 1.0 mL of stock solution (0.5% I_2 in 5.0% KI) into 500 mL of distilled water containing 5.0 mL of 5 N HCl . For the assay, the reaction mixture consisting of 0.1 mL of enzyme and 0.2 mL of soluble starch were incubated at 30 °C for 5 min. The reaction was stopped by adding 5.0 mL of iodine reagent. The absorbance was measured at 620 nm against at a

blank [24]. One unit of the α-amylase activity was defined as the amount of enzyme that hydrolyses 0.1 mg of starch, per min, under the assay conditions while the enzyme productivity has been expressed as U/g of dry substrate (U/gds).

The remaining glucose concentrations in the media were determined by the o-toluidin method [25]. To 0.5 mL of filtrate in test tubes, add 4.5 mL of *o*-toluidine reagent (dissolve 1.5 g of thiourea in 940 mL of glacial acetic acid and add 60 mL of *o*-toluidine). Place test tubes in boiling water for 10 min, cool with cold water; add 5 mL of glacial acetic acid. The absorbance was measure at 630 nm. The percentages of glucose in samples were calculated by the following formula;

$$\text{Glucose (mg/100mL)} = \frac{\text{Sample Absorbance}}{\text{Standard Absorbance}} \times 100$$

All the experiments were conducted in triplicate and the mean of the tree with standard deviation (SD) was represented.

Conclusion

SSF process using WB and RB were resistant to CR but BB culture was not. We can say that the reason for this is water holding capacity of the substrates WB and RB are greater than BB. This study is thought to be helpful for the production of α-amylase from *P. brevicompactum* in WB, RB and BB cultures which have high concentration of glucose.

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