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Optimization of Trehalase in *Aspergillus niger*

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ABSTRACT

Aspergillus niger produces enzyme trehalase, which is responsible for conversion of trehalose to glucose. Conidia from 15-day old culture were used for optimizing the trehalase activity using different cellulosic media and different carbohydrates; further its distribution was studied. Enzyme activity was assayed by estimating glucose released using 3,5-Dinitrosalicylic acid. Highest trehalase activity was reported in presence of oatmeal as compared to corn and walnut sources. Further, highest trehalase activity was present as extracellular protein. High levels of enzyme activity were obtained in presence of 1 % starch. There was nearly 80% increase in specific activity of the enzyme in presence of starch. Specific activity of trehalase enzyme in presence of starch was 46.62 ± 1.39 µmol/min/mg.

Key words: *Aspergillus niger, Conidia, Optimization, Trehalase.*

INTRODUCTION

Trehalase (α,α-trehalose-1-C-glucohydrolase, EC 3.2.1.28) is a glycoside hydrolase, an enzyme which catalyzes the conversion of trehalose to glucose. Trehalases exist in many prokaryotic and eukaryotic cells including bacteria[1], fungi[2], and higher plants[3], as well as in insects[4,5] and mammals[6]. Trehalase has been purified and characterized from various organisms such as *Saccharomyces cerevisiae*[7], *Lentinula edodes*[2], from the seeds of *Cicer arietinum*[8].

Only trehalase is responsible for trehalose utilization[9,10]. Trehalose hydrolysis by trehalase is important in various fungal physiological processes such as fungal spore germination and the resumption of growth in resting cells. Fungal cells accumulate trehalose up to 30% of the cell dry mass in response to stressful conditions like heat shock and water loss or during growth restriction due to an imbalance between carbon and nitrogen availability[11,12]. Trehalose is also a storage carbohydrate, accumulating under conditions of carbon deficiency in fungi[13]. Several *in vivo* experiments have shown that trehalose levels closely correlate with stress resistance. Some *in vitro* experiments have concluded that trehalose is a stabilizing agent of cell membranes and proteins as this sugar could substitute for water to prevent destabilization of lipid bilayers[14]. Trehalase is also responsible for in vivo growth and virulence of the entomopathogenic fungus, *Metarhizium acridum*[15].

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A number of *Aspergillus* species are known, but *Aspergillus fumigatus, Aspergillus flavus, Apergillus terreus* and *Aspergillus niger* are commonly associated with invasive infection in humans[16,17]. In humans *Aspergillus niger* can cause otomycosis[18]. *Aspergillus niger* is also causative agent of a disease called black mould on fruits and vegetables. Trehalose hydrolysis by trehalase is responsible for spore germination in fungal species[19], it is likely that by studying the characterization of the enzyme, one may learn to control the germination and thus prevent food spoilage.

MATERIALS AND METHODS

Microorganism and culture conditions

Aspergillus niger NS2 was maintained on 50ml media slants [4% (w/v) cellulosic media and 1.8% (w/v) agar] at 28 °C for 10 days then shifted to 4 °C for 5 days.

Purification of Trehalase from *Aspergillus niger*

Conidia from 15 day old culture were harvested. To 5 g spore paste of conidia , 10 g of alumina was added and cell suspension was prepared using 0.01 M sodium citrate buffer containing 1 mM phenylmethanesulfonyl fluoride. This suspension was ruptured using glass beads. The homogenate was centrifuged and resultant paste was suspended in 200 ml of 0.01 M sodium acetate buffer and was centrifuged. To the supernatant solution, 1 M acetic acid was added and the pH was adjusted to 4.0. The precipitate formed was removed by centrifugation. The supernatant was cooled to 0 °C, and equal amount of cold acetone was added. The precipitate was collected and dissolved in 10 ml of sodium acetate buffer, and dialyzed against 0.01 M phosphate buffer. The resultant solution adsorbed on Diethylaminoethyl (DEAE)-cellulose column which had been equilibrated with 0.01 M phosphate buffer. The enzyme was eluted by applying gradient of 0.05 to 0.3 M NaCl. All purification steps were carried out at 4 °C. At each step of purification, trehalase activity and protein content were determined using Bradford method (1976)[20].

Assay of trehalase

Trehalase activity was assayed using 3,5-Dinitrosalicylic acid method by Miller (1959)[21]. The reaction mixture contained 100 mM trehalose, 50 mM sodium acetate buffer (pH 5.0), and 0.25 ml enzyme extract in a final volume of 0.5 ml.

Optimization of Trehalase activity on different cellulosic sources

For optimizing trehalase activity, fungal culture was grown on three different cellulosic media containing 4% (w/v) of corn, oatmeal and walnut respectively for 15 days. Then relative weight of conidia and protein concentration was measured.

Distribution of Trehalase activity in *A. niger*

Distribution of trehalase was checked by studying the enzyme activity at various steps of purification, by checking the enzyme activity in cell extract after cell lysis.

Substrate Specificity

Studies relating to the induction of the enzyme activity were conducted using mycelia grown in a culture medium containing glucose as the main carbon source. After 48 h growth, mycelia was harvested, washed with sterile distilled water and transferred to 25 ml fresh medium supplemented with 1 % each of glucose, trehalose, starch, sucrose, xylose, fructose or mannose respectively.

Statistical analysis

The statistical analysis of data was done using paired Student's t-test. P-value ≤ 0.05 were considered significant

RESULTS

Optimization of Trehalase activity on different cellulosic sources

For the selection of best source of cellulose, fungal culture was grown on three different cellulosic media containing 4% (w/v) of corn, oatmeal and walnut respectively for 15 days. Then relative weight of conidia and protein concentration was measured, these results are represented in Figure 1 and 2. It is apparent that dry weight of conidia was quite high (0.63 ± 0.05 g/100ml) in presence of oatmeal as compared to that from corn (0.234 ± 0.03 g/ml) or walnut (0.186 ± 0.02 g/ml)

respectively grown cultures. And the protein concentration in conidia grown on oatmeal was 8.62 ± 0.24 mg/g dry weight which was higher than in cultures grown containing corn (1.7 ± 0.10 mg/g dry weight) or walnut (1.4 ± 0.15 mg/g dry weight). So for further studies oatmeal was used as source of cellulose for growth of *Aspergillus niger*.

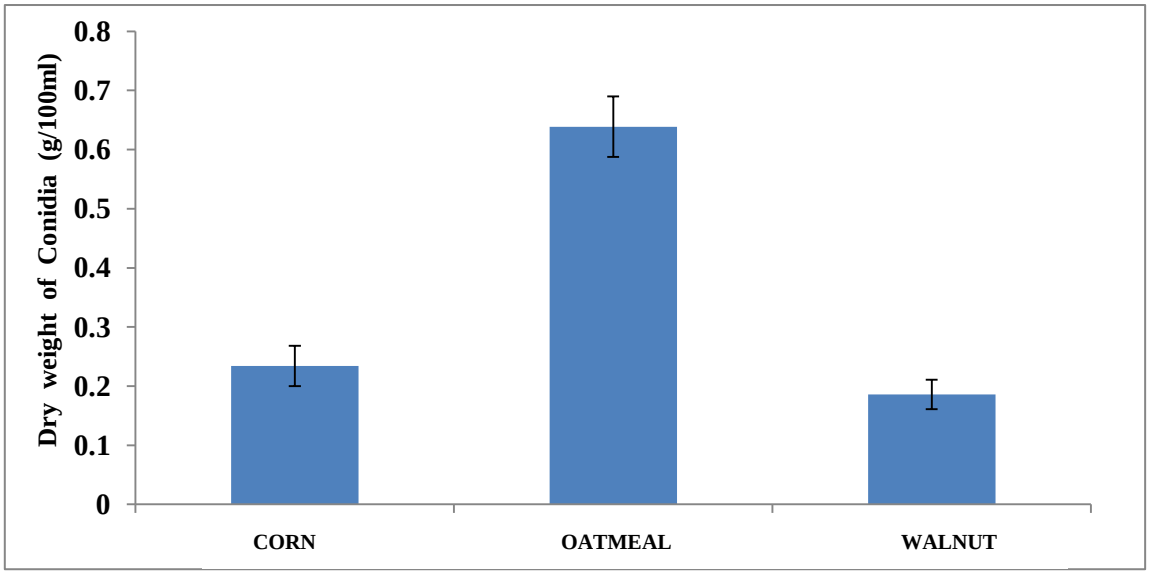

Fig. 1: Dry weight of *Aspergillus niger* conidia grown on different carbon sources expressed as g/100ml. Data is the mean of three independent experiments ± standard deviation, indicated by bars, **p ≤ 0.001. Details are mentioned in Materials and Methods section

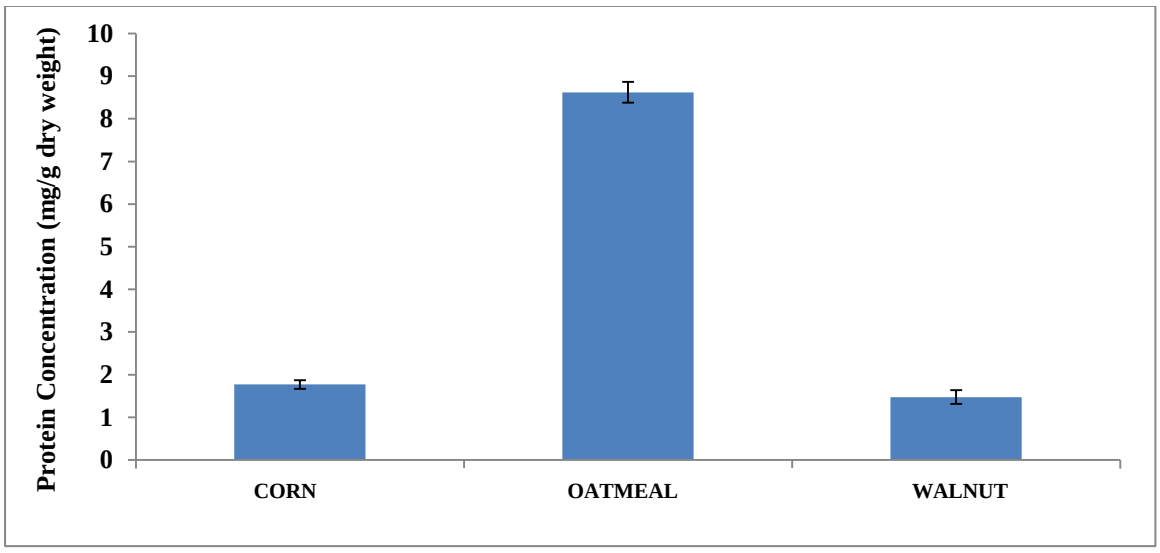

Fig. 2: Protein content of conidia grown on various cellulosic sources. Protein concentration in conidia, expressed as mg/g dry weight of conidia. Data is the mean of three independent experiments ± standard deviation, indicated by bars. ***p ≤ 0.001

Distribution of Trehalase activity in *A. niger*

The distribution of trehalase activity was further checked in *Aspergillus niger*. As shown in Table 1, enzyme activity was mainly located as an extracellular protein. Cell associated trehalase activity was much lower (16%) as compared to that present in extracellular medium (77%). 7% of activity was present in the pellet which was collected after the cell lysis, carried out by glass beads. Thus, extracellular protein was selected as the source of enzyme trehalase for further studies and enzyme purification.

Table 1: Distribution of trehalase activity in *A. niger:* 50 ml of the cell culture was centrifuged at 10,000 × g for 10 min. Trehalase activity was measured in the supernatant and pellet fractions. (Values are Mean ± SD, n=3)

Preparation	Activity (µmol/min/ml)	Percentage Activity (%)
Extracellular	0.182 ± 0.006	77.11
Intracellular	0.037 ± 0.005	15.60
Pellet	0.017 ± 0.004	7.20

Effect of carbon source on trehalase production by *A. niger*

Several sugars were tested for trehalase production by *Aspergillus niger*. After 48 h growth, mycelia was harvested, washed with sterile distilled water and transferred to 25 ml fresh medium supplemented with 1 % of each carbon source. High levels of enzyme activity were obtained in presence of starch. There was nearly 80% increase in specific activity of the enzyme in presence of starch. Specific activity of trehalase enzyme in presence of starch was 46.62 ± 1.39 µmol/min/mg. In contrast, only moderate levels of total enzyme units were secreted into the medium when trehalose (which is the innate enzyme substrate) was added. Specific activity of trehalase was 23.23 ± 2.88 µmol/min/mg in presence of trehalose which is little less than the culture deficient of carbon source. Glucose, Fructose and Mannose slightly enhanced the trehalase activity by approximately 5 units/mg of Specific activity. Whereas, Sucrose and Xylose decreased the enzyme activity abruptly showing decrease in specific activity by 31% and 62% respectively. Specific activity in presence of Sucrose was 18.05 ± 1.39 µmol/min/mg and in presence of Xylose it was 10.23 ± 2.03 µmol/min/mg (Table 2).

Table 2: Effect of various carbohydrates on enzyme production: After 48 h growth, mycelia was harvested, washed with sterile distilled water and transferred to 25 ml fresh medium supplemented with 1 % of each carbon source (Values are Mean ± SD, n=3)

Carbon Source	Total Units (per ml)	Total Protein (mg) (per ml)	Specific Activity (µmol/min/mg)
Deficient	59.48 ± 2.50	2.29 ± 0.17	25.93 ± 1.00
Glucose	67.11 ± 0.80	2.18 ± 0.12	30.80 ± 1.43
Trehalose	55.59 ± 0.83	2.41 ± 0.25	23.23 ± 2.88
Starch	96.59 ± 0.83	2.07 ± 0.04	46.62 ± 1.39
Sucrose	46.21 ± 1.20	2.57 ± 0.23	18.05 ± 1.39
Xylose	25.58 ± 0.61	2.55 ± 0.45	10.23 ± 2.03
Fructose	66.58 ± 0.83	2.27 ± 0.12	29.38 ± 1.79
Mannose	61.48 ± 1.20	2.10 ± 0.10	29.30 ± 1.80

CONCLUSION

In conclusion, trehalase activity is highest when oatmeal is used as cellulosic media. Trehalase activity is mainly distributed as extracellular protein. And starch strongly enhances the trehalase production as compared to other carbohydrates such as Glucose, Trehalose, Sucrose, Xylose, Fructose and Mannose. So by further characterizing the trehalase enzyme we can control its activity, further controlling spore germination and thus prevent food spoilage and control infections caused by *A. niger*.

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