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Trehalose Production by a Starch Assimilating Yeast *Cryptococcus aerius*

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Abstract: Production of trehalose by an isolated starch assimilating yeast *Cryptococcus aerius* under some conditions such as different concentration of carbon source, temperature stress, oxygen limitation and various nitrogen sources was investigated. Trehalose production was favorable in a medium contained 1% starch; in a time course about 72 h at 30°C. It was proved that the starch degradation rate and reducing sugars concentration in medium were the factors that controlled trelahose accumulation in all conditions. Cultivation of *C. aerius* at 37°C was a stress condition for this yeast, but because of low starch degradation rate at this temperature it was not an optimum condition for trehalose accumulation. Due to high rate of starch degradation and assimilation by *C. aerius* in the presence of inorganic nitrogen source, trehalose accumulation rate improved relative to using complex organic nitrogen source such as yeast extract. These results indicate that trehalose production can be conducted efficiently by using starch as carbon source and a yeast like *C. aerius*.

Key words: *Crptococcus* sp., starch, amylase, trehalose, nitrogen

INTRODUCTION

Trehalose, a non-reducing disaccharide (α-D-glucopyranosyl-α-D-glucopyranoside) considered to play important physiological functions in a variety of bacteria and fungi and in some plants and animals, in addition in spores, cysts, sclerotia and dried resurrection plants[1-3].

Trehalose is commonly known to act as a storage carbohydrate and accumulates as the cells enter the stationary growth phase. Furthermore, intracellular trehalose is mobilized during periods of growth condition, including that the compound may serve as a carbon source[4]. However it has been suggested that trehalose also functions in yeast as a protectant that contribute to survival during environmental stress condition such as heat, freezing, dehydration, desiccation and exposure to toxic chemicals[1,4,5].

Tow hypotheses (the water replacement and the glass transition hypotheses) have been proposed to explain the protective effects of trehalose on cells, but there is no straight forward explanation[1].These unique protective properties of trehalose make it an interesting compound for several application, e.g. as cryoprotectant for cell in medicines and microbiology, as an effective component in cosmetic, as a stabilizer for clinical reagents and by products and even as a preservative for fresh foodstuff[1,2,3,5]. Various methods of industrial production of trehalose have been available. They include: 1) production of trehalose from starch or maltose by thermostable enzymes from thermophilic bacteria. 2) accumulation of trehalose in cells using *S. cerevisiae* and basidiomycotina yeasts. 3) Synthesis of trehalose from glucose-1-P by trehalose phosphorylase from *Pichia fermentanus*[1,6].

It is reported that trehalose produced by enzymatic conversion can be applied to food industries as the final reaction system contains other sugars in addition to trehalose while trehalose produced by the cellular accumulation due to its purity, can be applied to medical industries[1].

It is thought that the best substrate for production of trehalose is starch due to its low price and high carbon source[1]. The aim of this study was to investigate the ability of this isolated hyper-amylase producing yeast for trehalose accumulation in various conditions.

MATERIALS AND METHODS

Yeast strain: An amylolytic yeast was isolated from wastewater of a corn starch factory by the method of Kerger-van Rij[7,8]. It was identified based on the biochemical and morphological tests[7]. The ability of the yeast to hydrolyze starch was tested though starch assimilation test on solid growth medium (1.5% agar) by the auxanographic method[8,9]. Yeast maintained on yeast extract glucose chloramphenicol agar (GYC from Merck Co.) at 4°C.

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Media: YPS medium, which contained 2% soluble starch and 1% yeast extract and 2% peptone, was used to cultivate the yeast cells on the agar slants.

SSY (soluble starch yeast extract) medium, which used for trehalose production, consists of soluble starch in different concentrations (0.5, 1.0, 1.5 and 2.5%) and 1% yeast extract, pH 5.5.

The third medium which was used for amylase assay and trehalose production was ASS (Ammonium sulfate starch) which contains: starch 1%, $(NH_4)_2SO_4$ 0.5%, K_2HPO_4 0.5%, KH_2PO_4 0.14%, $CaCl_2$ 1 H_2O 0.02%, $MgSO_4$ $7H_2O$ 0.05%, pH 5.5.
All the media were prepared based on w/v.

Enzyme assay: Yeast inocula were prepared by inoculating slant cultures to 100 mL of sterile ASS contained in 500 mL Erlenmeyer flasks. The flasks were incubated with shaking (200 rpm) at 30°C for 36 h. Three replicate flask (500 mL) contained 140 mL ASS media (with 0.5% soluble starch) were inoculated with 10 mL of inocula and maintained in the same condition described for inocula preparation. At regular time intervals, samples were taken and total extracellular amylase activity was tested in its supernatant. Total extarcellular amylolytic activity was measured by the 3,5-dinitrosalicylic acid (DNS)[10,11]. A standard curve for this colorimetric assay was constructed, using glucose as the standard. One unit of amylolytic activity (U) was defined as the amount of enzyme in 1 mL that liberates 1μmol of reducing sugar from soluble starch in 1 min at 30°C.

Trehalose accumulation in shaking culture: Seed cultures were prepared by inoculating cells grown on a YPS agar slant into a 250 mL Erlenmeyer flask that contained 50 mL of the SSY liquid medium with subsequent incubations at 30°C for 18 h with shaking (200 rpm). Ten milliliter of the feed culture was then transferred into a 250 mL flask that contained 90 mL of the ASS or SSY liquid medium which contained 0.5, 1.0, 1.5, 2.5 g soluble starch in 100 mL then they were incubated at 20, 30 and 37°C. The change in Oxygen Transfer Rate (OTR) in each flask achieved by changing shaking (rotation) speed.

Trehalose extraction with trichloroacetic acid (TCA) and its assay: The yeast cells from 5 mL culture was harvested and washed three times with ice cold distilled water by centrifugation at 4000 rpm for 10 min. Four milliliter of ice cold 0.5 M trichloroacetic acid (TCA) was added to the pellet of the yeast cells. The mixture was incubated at 0°C for 20 min with frequent shaking. The sample was centrifuged at 4000 rpm for 5 min and the supernatant was collected. After centrifugation the cell were extracted twice with another 4 mL of ice-cold 0.5 M TCA. The supernatant were combined to make up to 12 mL of the extract. After suitable dilution trehalose content in the extract was assayed by anthron reaction[1,5].

Analytical procedure: Unless otherwise mentioned, samples (10 mL) were collected from a given flask and centrifuged at 4000 rpm for 10 min to remove cells and supernatant fluid was used for determining residual starch concentration, glucose concentration, amylase activity and the pellet was used for biomass determination.

Measurement of cell dry weight: The yeast cells from 10 mL of culture was harvested and washed three times with distilled water by centrifugation at 4000 rpm for 10 min. Then, cells in the tube dried at 70°C until the cell dry weight was constant.

Measurement of residual starch: An iodine reagent was prepared fresh by diluting 1 mL of stock solution (0.5% I_2 in 5.0% KI) into 500 mL of distilled water containing 5 mL of 5N HCl. For the measurement, 5 mL of the iodine reagent was mixed with 0.2 mL of starch solution (With more than 1.0% starch solution, the starch solution was diluted ten times with distilled water) then the temperature was adjusted at 30°C in bathwater for 3-4 min and the absorbance was measured at 620 nm against a blank (0.2 mL of water in 5 mL of iodine reagent)[12].

Measurement of free glucose in medium: Free glucose in media was measured by enzymatic method (Glucose oxididase). A sample of medium was taken at time interval, then boiled to denatured all enzymes for 10 min, after that it was used for glucose determination.

Mean value of at least three separate experiments for identical flasks have been presented.

RESULTS AND DISCUSSION

Isolation and identification of amylase producing yeast: The starch hydrolyzing yeast which was isolated from corn starch processing industrial waste was identified by observation of morphological characteristics and by physiological tests such as fermentation, carbon assimilation, nitrogen assimilation, urease test, Diazonium blue B color test, etc. The isolated amylase producing yeast was an obligate aerobic yeast, that only assimilated sugar such as glucose, galactose, sucrose, maltose and

Table 1: The effect of glucose and starch residue on trehalose accumulation

Starch (%)	0.5			1.0			1.5			2.5		
Time (h.)	24	48	72	24	48	72	24	48	72	24	48	72
Glucose (mg/100)	0.32	0.02	0	0.29	0.4	0	0.52	0.12	0	0.37	0.16	0.12
Trehalose (%)[a]	0.1	4.2	3.03	1.13	8.6	10.1	0.17	3.2	6.2	0.15	2.4	2.9
Residual starch (g/100)	0.07	0	0	0.73	0.01	0	0.67	0.09	0.01	0.8	0.35	0.4

(The results are averages of three separated experiments), [a]- trehalose concentration is according to the cell dry weight

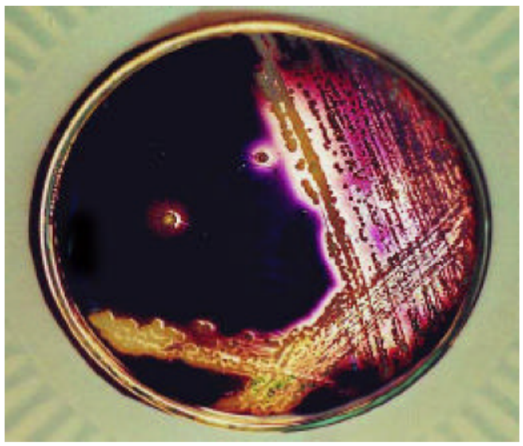

Fig. 1: Soluble starch hydrolyzing by *C. aerius* in growth medium at 30°C in 24 h

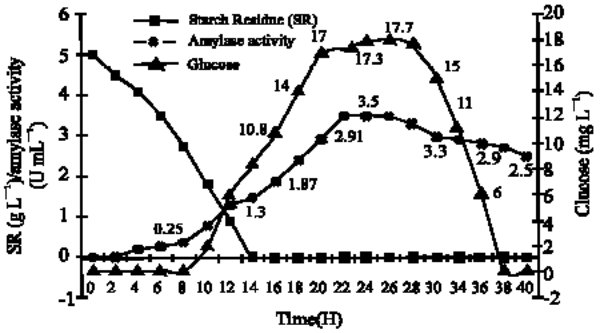

Fig. 2: Amylase production and starch degradation in ASS medium by *C. aerius*

starch. It wasn't able to ferment any sugar; it means that under anaerobic condition with 20 g L^{-1} of sugar it can not produce gas or acid[7]. It could assimilate nitrate and nitrite as nitrogen source and had a growth temperature range between 20-35°C but growth at 20 and 37°C was low. It could grow in vitamin free media; growth in vitamin free media is economically desirable for industrial application. Based on the above characteristics and the others the isolated yeast was identified as *Cryptococcus areius*. It has no pathogenic effect[7]. After isolation and identification it was tested for its potential to produce amylases on solid medium with soluble and raw wheat starch as carbon source. *C. aerius* showed a wide and clear hydrolysis halo on the growth medium (Fig. 1). Comparing the results of this study with pervious studies, shows that various specious of *Cryptococcus* when grown on starch-containing medium, exhibits high amylase production after 24 h of cultivation[13,14].

Amylase production by *C. aerius*: Preliminary experiments using various initial pH values (2-9) and different cultivation temperature had shown that amylase production by *C. aerius* was highest in a medium with initial pH between 4.5-6 at 30°C, therefore we selected 5.5 as the initial pH and 30°C as the optimum temperature for further investigation. In a another preliminary experiment the effect of starch concentration on starch degradation rate was tested, since it would be more desirable for industrial application to be able to utilize high substrate concentration, 10-70 g L^{-1} soluble starch was used in mono culture of *C.aerius*, the results showed that when starch concentration increased from 30 g L^{-1} the starch degradation rate decreased rapidly; since *C. aerius* is an obligate aerobic species and starch gelatinization after heat treatment increases, so probably by increasing in starch concentration, the oxygen transfer rate decreases and as the result starch degradation rate.

A typical pattern for the time course of starch degradation and amylase production (Fig. 2) showed that starch hydrolysis proceeded quickly during the early exponential phase but low level of reducing sugars were detected. Starch degraded in the early stage of cultivation and a rapid loss of iodine staining was found but at this time there was a low level of extracellular amylase in media (Fig. 2); this probably reflects the involvement of cell-bound activity in early starch hydrolysis. Glucose production proceeded after the starch degradation and simultaneously by assimilation of reducing sugar, biomass increased rapidly, it was demonstrated that probably there was a negative effect of reducing sugar (e.g. glucose) on amylase production and while glucose increased in media amylase secretion decreased. According to pervious studies on amylase secretion patterns by various yeasts, three different patterns have been described for extracellular amylase production[15]; a rapid loss of iodine-staining combined with a high final

Table 2: Effect of temperature on trehalose accumulation in SSY medium in 200 rpm after 96 h

	Temperature (°C)		
	20	30	37
Starch residue[a] (g L^{-1})	0	0	0
Glucose(mg/100)	0.81	0	0.57
Trehalose (%)	1.93	10.1	1.52
Biomass (g L^{-1})	3.58	4.22	1.81

[a]-Initial starch concentration was 1%

Table 3: Effect of nitrogen source on starch degradation and trehalose production in ASS and SSY medium

	Medium					
	ASS[a]			SSY[b]		
Time course (h)	24	48	72	24	48	72
Starch residue(%)[c]	0.02	0	0	0.73	0.01	0
Trehalose (%)	1.21	9.85	9.9	1.13	8.6	10.1
Biomass (g L^{-1})	2.8	4.18	4.33	1.92	2.92	4.22

[a]-Ammonium sulfate starch media [b]-Soluble starch yeast extract media [c]-The initial starch concentration was 1% and cells were incubated at 30°C in 200 rpm

level of reducing sugar probably indicated the presence of both α-amylase and glucoamylase for *C. aerius*.

Effect of different concentrations of soluble starch on trehalose accumulation: The results indicated that trehalose content in cell was increased when soluble starch concentration in the medium increased from 0.5 to 1.0% (Table 1). For example, when soluble starch concentration in the medium was 1.0%, trehalose content per 100 g cell dry weight was 10.1 g, however when soluble starch concentration in the medium was increased from 1 to 2.5%, trehalose content was decreased rapidly; although cell dry weight increased. These can be explained with the fact that reducing sugars concentration in medium is critical for trehalose production and accumulation (Table 1), when starch degradation is complete and most of reducing sugars (produced from starch degradation) are assimilated by *C. aerius*, the cells faces with a starvation mood, that makes them to accumulate trehalose as stress response to carbon starvation. It was reported that it is very important to keep the low level of reducing sugar in order to make yeast cells accumulate higher trehalose concentration, as higher reducing sugar can induce the activation of neutral trehalase in yeast cells which is responsible for hydrolyzing trehalose in yeast cells[16].

On the other hand trehalose accumulation was dependent to cultivation time (Table 1); maximum accumulation of trehalose was reached when the yeast cells were grown for >72 h. It also showed that when cell growth reached early stationary phase, they accumulate the highest amount of trehalose. In fact at stationary phase when cells starve (glucose concentration is very low) they began to increase their storage such as trehalose.

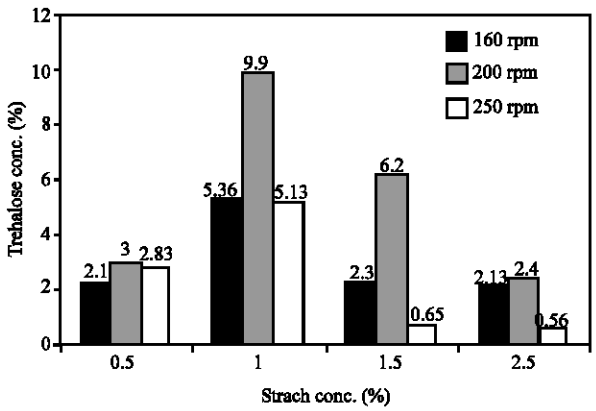

Fig. 3: Effect of OTR on trehalose accumulation by *C. aerius* in 72 h at 30°C

Effect of Oxygen Transfer Rate (OTR) on trehalose accumulation: To investigate whether the oxygen level in medium can influence the trehalose accumulation, three different rotation rate were used. Trehalose accumulation by this yeast was adversely affected at high and low OTR (Fig. 3). We observed maximum level of trehalose in a medium which was shaken at 200 rpm for 72 h. However, shaking at 250 and 160 rpm due to low biomass production and low starch degradation rate, respectively cannot support high level of trehalose accumulation.

Effect of temperature on trehalose accumulation: As a rule, trehalose accumulation is high under stress conditions[1,4,16]. Three temperature, 20, 30 and 37°C were chosen because 30°C is favorable temperature for degradation by this yeast while 20 and 37°C is critical temperature for growth of this yeast. Trehalose accumulation is high, in SSY medium, only at 30°C (Table 2). As mentioned before, starch and glucose concentration were critical factors that permited cells to enter stationary phase and subsequently biosynthesis of trehalose so when starch degradation was slow at 20°C and growth isn't optimum at 37°C, even after 96 h the starch remained in media.

Effect of inorganic nitrogen source on trehalose accumulation: To investigate the effect of inorganic nitrogen source on trehalose accumulation, yeast extract in SSY was substituted with 0.5%w/v $(NH_4)_2SO_4$ in ASS medium. There was no significant effect on trehalose accumulation and in 1% starch concentration at 30°C and 200 rpm the trehalose was 9.85% (Table 3), but because of

higher rate of starch degradation (in the presence of inorganic nitrogen source) the time course for arriving at stationary phase is shorter than using yeast extract as the sole nitrogen source, so it is suggested that inorganic nitrogen source is used instead of inorganic nitrogen source (yeast extract).

The results of this investigation clearly show that starch can serve as an excellent carbon source for trehalose production by *C. aerius*. Further investigation should be designed to improve trehalose production by this yeast.

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