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Role of ethanol and calcium towards the level of glycogen and surface α-glucans as a measure of flocculation in *Saccharomyces italicus*

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ABSTRACT

Apart from soluble and insoluble pool of glycogen ethanol also affects the third pool of glycogen present at the cell surface level of yeast. α-glucans which play an important role in the process of yeast flocculation also contribute to this third pool of glycogen in yeast cells as confirmed by amyloglucosidase treatment. Cells grown in the presence of ethanol exhibit higher amount of surface α-glucans indicating the role of ethanol in enhancing flocculation in yeast. Addition of Ca^{2+} as well as both Ca^{2+} and ethanol collectively to yeast culture medium exhibits both increase in cell wall bound insoluble glycogen content and surface α-glucans. Ethanol action on yeast cells also exhibits rise in the content of cell surface glycoprotein which includes glucomannoproteins and galactomannoproteins present in the outer layer of cell wall. Thus yeast cells exhibit increase in the level of cell wall bound insoluble glycogen, surface α-glucansas well as surface glycoprotein as a protective measure against ethanol stress. Thus both Ca^{2+} and ethanol enhance flocculation in yeast cells where the level of flocculence is correlated with the cell surface α-glucans.

Introduction

Yeast cell envelope consists of cell wall, periplasmic space and plasma membrane. It has a major role in maintaining osmotic homeostasis and permeabilty properties of the cell. Cell wall in *S. cerevisiae*, is a remarkably thick envelope which contributes to 15 to 25% of the dry mass of the cell. The combination of considerable mechanical strength and high elasticity allows the wall to transmit and redistribute physical stress, thus offering efficient protection against mechanical damage (10). Major structural constituents of the cell wall includes polysaccharides (80- 90%); mainly glucans and mannannoproteins, with a minor percentage of chitin and lipids (6). Inner layer of load-bearing polysaccharides of cell wall which form a microfibrillar network includes both β-1, 3 and β-1, 6-linked glucans providing strength to the cell wall. Cell wall glycogen in *Saccharomyces cerevisiae* was found to be attached to β-glucan component (2). Cell wall proteins of *Saccharomyces cerevisiae* can be divided into two groups as SDS extractable proteins and the remaining as mannoproteins released after β-(1-3) glucanase treatment, indicating that they are intimately associated with cell wall glucan (17). Glucanase-extractable wall proteins not only carry N- and O-linked side chains, but also glucose containing side chains (18). In particular, the diversity of the protein population that is anchored to the stress-bearing polysaccharides of the cell wall plays an important role in adaptation of the cell to environmental conditions, in growth mode and in survival. Role of cell surface α-glucans in flocculation of yeast is confirmed by amyloglucosidase treatment (1). Calcium ions also exhibit an important role in flocculation over a broad pH range (16) as well as in lectin mediated flocculation mechanism by maintaining these lectins in active conformation. The surface of yeast cells plays a major role in aggregation, flocculation and adhesion, which are important for the production of bright beer. The mechanism by which yeast cells flocculate involves surface proteins on flocculent yeast cells binding to carbohydrate receptors on nearby cells (13). Lectin mediated flocculation mechanism requires Ca^{2+} ions to maintain their active conformation (9). Calcium ions are required for mutual adhesion of cells by forming salt bridges between their surface carboxyl groups (7, 14). Flocculation is an inheritable characteristic of yeast cells which includes FLO genes like FLO1, FLO2, FLO4, FLO5 and FLO8 (3). Flocculation level in yeast is determined by FLOP, a flocculation protein at the cell surface (8). Mechanism of flocculation in top and bottom flocculating yeast cells is different where the important determinant of flocculation in *S. cerevisiae* involves cell surface hydrophobicity, surface charge and zymolectin density. Ethanol enhances flocculation in top fermenting yeast cells while flocculation in bottom fermenting yeast is mediated by lectins along with Ca^{2+} ions.

So the studies will be carried out regarding the effect of ethanol and Ca^{2+} on cell surface carbohydrate, surface protein as well as flocculation in *Saccharomyces italicus*. This will conclude the correlation between flocculation, cell wall glycogen and cell surface α-glucans in yeast cells.

Materials and methods

Microorganisms & Cultural conditions:

Bottom flocculating yeast strain of *Saccharomyces italicus* NCIM-3230 was from National Chemical Laboratory, Pune. All the strains were routinely maintained on YEPD agar at $+4^0$C.

Peptone, yeast extract, malt extract were from Difco Laboratory, Detroit, MI. Amyloglucosidase was purchased from Sigma Chemical Company; USA & Glucose Oxidase Kit was from BioLab. Cerric ammonium nitrate was from Qualigen.

Glucose was measured using glucose oxidase peroxidase method (11) and total carbohydrate was estimated by phenol sulfuric acid method (5). Proteins were analyzed by Folin Lowry method (12).

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Media composition:

Media with variable concentrations of glucose and ethanol were used to study their effect on the glycogen, cell surface α-glucans, surface carbohydrate as well as surface proteins in yeast cells.

Set A: Increasing sugar concentration (Flasks: a, b, c, d, and e)

2, 4, 6, 8 and 10% glucose; 0.5% peptone and 0.3% yeast extract in a set of five flasks each.

Set B: Increasing sugar concentration and constant ethanol concentration (Flasks: f, g, h, i, j)

2, 4, 6, 8, 10 % glucose, 0.5% peptone, 03% yeast extract and 8 % (v/v) ethanol in a set of five flasks.

Set C: Effect of $CaCl_2$ on yeast glycogen content:

YPG: (0.3 % yeast extract, 0.5 % peptone, 8 % glucose).

YPD-LCa: (0.3 % yeast extract, 0.5 % peptone, 8 % glucose, 0.08 % $CaCl_2$).

YPD-HCa: (0.3 % yeast extract, 0.5 % peptone, 8 % glucose, 0. 8 % $CaCl_2$).

Set D: Effect of $CaCl_2$ and ethanol on yeast glycogen:

YPDE: (0.3 % yeast extract, 0.5 % peptone, 8 % glucose, 8 % v/v ethanol).

YPD-LCaE: (0.3 % yeast extract, 0.5 % peptone, 8 % glucose, 0.08 % $CaCl_2$, 8 % v/v ethanol).

YPD-HCaE: (0.3 % yeast extract, 0.5 % peptone, 8 % glucose, 0.8 % $CaCl_2$, 8 % v/v ethanol).

Harvesting of yeast cells

All the flasks from Set A, Set B, Set C and Set D were inoculated with 24 h starter culture (10 % v/v) grown in the same medium. Fermentation was carried out at room temperature (25 °C). Then yeast cells were harvested under cold conditions from each flask after 48 h of fermentation and washed repeatedly with chilled distilled water.

Isolation of glycogen

Glycogen was isolated from yeast cells by alkali digestion method (15). Cells harvested from culture medium were subjected to alkali digestion using 2 ml 20 % KOH/g yeast cells and kept in boiling water bath for 1h. The resultant alkali digest was cooled in ice bath and adjusted to pH 7.0 using ice cooled 0.5 M HCl. The yeast cell digest was centrifuged to separate out supernatant containing soluble glycogen and the residual insoluble jelly like mass consisting of cell wall β-glucans. Both these fractions were used further for quantitative determination of soluble and insoluble glycogen as well as total carbohydrate.

Estimation of glycogen: 1 mL volumes of the digest supernatant and residual sediment were incubated with 1.2 ml solution containing sodium acetate buffer, pH 4.2, amyloglucosidase (2 I.U.) and α-amylase (1 I.U.) at 37 ºC for 1 h. Glucose released in the reaction mixture was measured by glucose oxidase peroxidase method. The amount of glucose in the supernatant and residual sediment represents soluble and insoluble glycogen respectively.

Determination of surface α-glucan content

Yeast cells harvested from from set A, B, C and D were subjected to amyloglucosidase treatment to determine cell surface α-glucans as well as surface protein content.

Cells were fixed by using 1.5 % v/v glutaraldehyde. 1 g yeast cells suspended in 4 ml of sodium acetate buffer (100 mM, pH 4.2) were then treated with 0.3 ml of amyloglucosidase at 37 ºC for 1 h. Along with the sample tube an appropriate control was also prepared. The reaction mixture was centrifuged and the supernatant obtained was used for estimation of cell surface α□-glucan as well as total carbohydrate content by the glucose oxidase peroxidase method and phenol sulphuric acid method respectively.

Determination of Total carbohydrate

Fractions of supernatant solution and residual suspension derived by centrifugation of neutralized yeast digest contained soluble and insoluble carbohydrate respectively. Total carbohydrate was measured by phenol sulphuric acid method.

Results

Presence of a third pool of glycogen at the cell surface level of yeast represents surface α-glucans made up of glucose residues predominantly is confirmed by amyloglucosidase treatment.

From Figure 1 it exemplifies those cells grown in media containing both glucose (2-10 % w/v) as well as ethanol (8% v/v) display higher surface α-glucan content than those observed for the cells grown in media with only glucose (2-10 w/v). This indicates that exracellular ethanol not only affects the storage level of cytoplasmic & cell wall bound insoluble glycogen (4) but also third pool of cell surface glycogen present in the form of α-glucans.

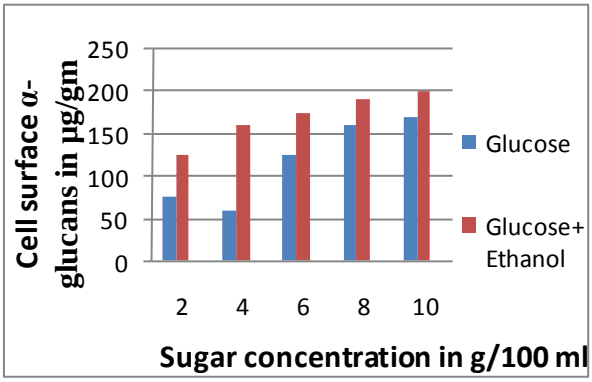

Figure 1. Amount of surface α-glucans released after amyloglucosidase treatment

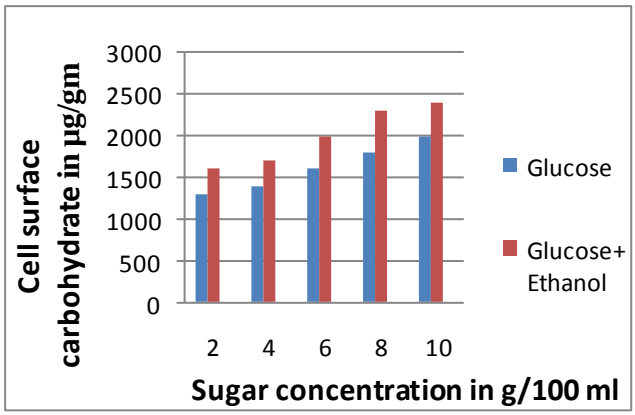

Figure 2. Amount of cell surface carbohydrate released from yeast cells after amyloglucosidase treatment

Cell surface carbohydrate in yeast cells comprised of glucose and mannose presenting mannan side chains as well as galactose. Figure 2 indicates the similarity of the results regarding total carbohydrate content with those obtained for surface α-glucans in yeast cells. The carbohydrate content was found to be higher when yeast growth media contained 8 % v/v ethanol in addition to glucose (2-10 % w/v).

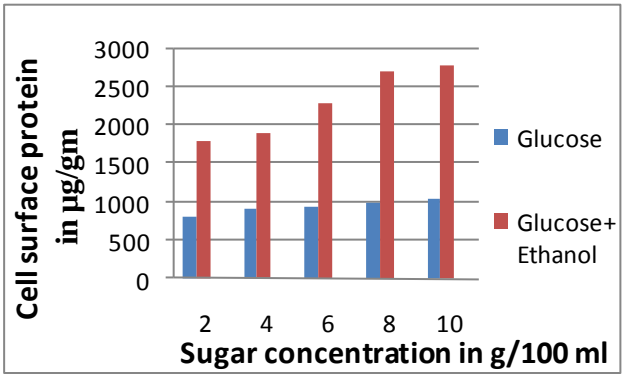

Figure 3. Amount of cell surface proteins released from yeast cells after amyloglucosidase treatment

From Figure 3 it is observed that cells grown in the medium containing 8% (v/v) ethanol in addition to glucose (2-10 % w/v sugar) exhibit comparatively higher surface protein content than those obtained for yeast cells grown in glucose (2-10 % w/v sugar) medium. Thus action of amyloglucosidase on yeast releases both the glucose as well as proteins present at the cell surface level.

Effect of $CaCl_2$: The effect of $CaCl_2$ on glycogen and carbohydrate content of yeast cells is depicted in Figures 4 and 5. The amount of insoluble glycogen (7.6) and carbohydrate (22.5 mg) in yeast cells from medium YPD-LCa containing 0.8 g/L $CaCl_2$ was similar as in cells grown in YPD medium without CaCl2 (7.4 mg glycogen and 23 mg carbohydrate). Cells grown in the medium YPD-HCa containing excess of $CaCl_2$ (8 g/L) exhibited decrease in insoluble glycogen (4.5 mg) and carbohydrate (18.0 mg) content. Higher amount of surface α-glucans were observed for yeast cells from both YPD-LCa and YPD-HCa media containing $CaCl_2$. (Table-1).

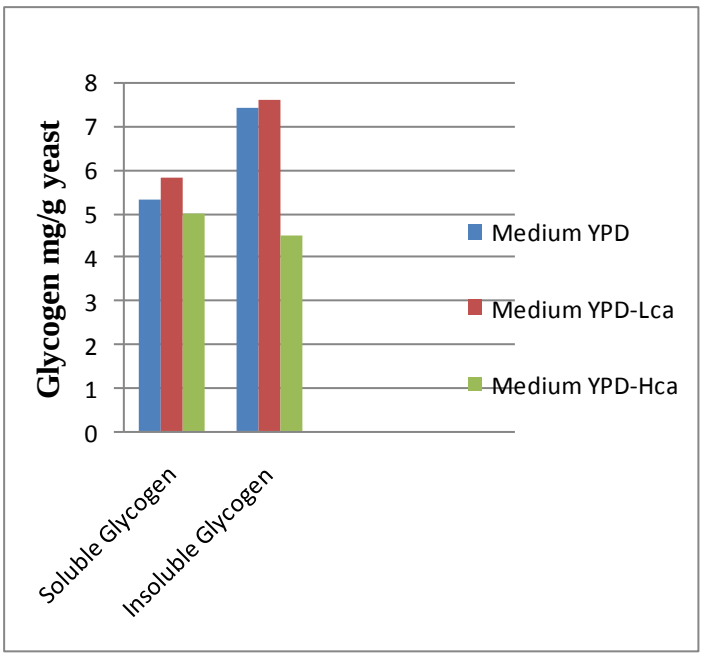

Figure 4. Effect of $CaCl_2$ on yeast glycogen content

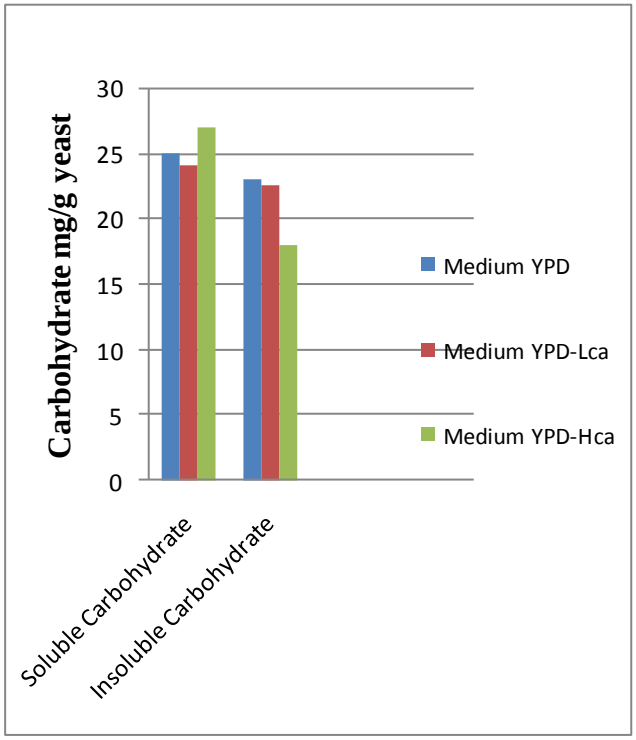

Figure 5. Effect of $CaCl_2$ on yeast carbohydrate content

Table 1. Effect of $CaCl_2$ on Surface α-glucan content of yeast cells

Media	Surface α-glucan (µg/g) wet wt.	Media	Surface α-glucan (µg/g) wet wt.
YPD	160	YPD-E	190
YPD-LCa	240	YPD-LCaE	295
YPD-HCa	290	YPD-HCaE	365

Effect of $CaCl_2$ and Ethanol: Combined effect of $CaCl_2$ and ethanol on yeast glycogen content is displayed in Figure 6 and 7. Yeast cells grown in the medium YPD-LCaE containing 0.08 g/L $CaCl_2$ and 8 % (v/v) ethanol exhibit higher amount of insoluble glycogen (11.4 mg/g) and carbohydrate (43 mg/g) than those for the cells grown in YPD medium. But the amount of both glycogen and carbohydrate was reduced 6.7 mg and 31.0 mg when the YPD-HCaE medium with excess amount of $CaCl_2$ (0.8 g/L) along with 8 % (v/v) ethanol was utilized for yeast cell growth. Cells harvested from media YPD-LCaE and YPD-HCaE also exhibited higher amount of cell surface α-glucans as 295 µg and 365 µg respectively as a result of combined effect of $CaCl_2$ and ethanol on yeast (Table 1). No significant increase was observed in the respective values of soluble glycogen and carbohydrate content.

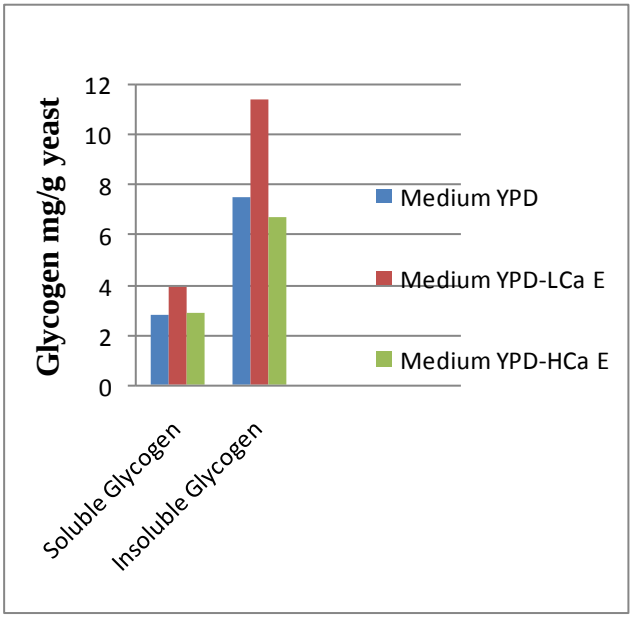

Figure 6. Effect of $CaCl_2$ and Ethanol on yeast glycogen content

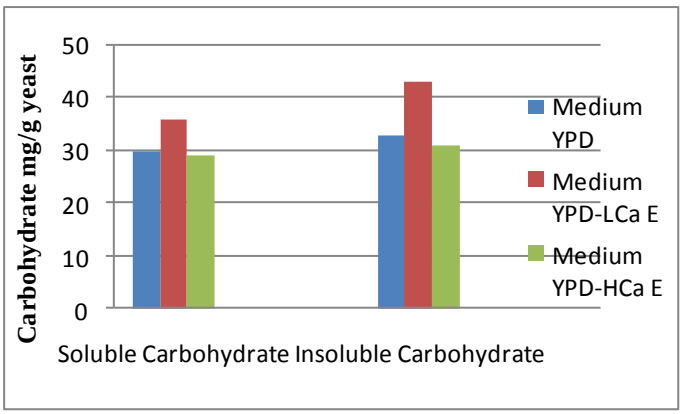

Figure 7. Effect of $CaCl_2$ and Ethanol on yeast carbohydrate content

Discussion-Conclusion

Yeast glycogen is comprised of α-glucans with α (1, 4) and α (1, 6) linkages and it serves as a major storage carbohydrate. Presence of third pool of glycogen in the form of α-glucans at the cell surface level of *Saccharomyce italicus* is confirmed by amyloglucosidase treatment. Ethanol plays an important role in the flocculation process of these cells thereby showing higher amount of insoluble glycogen as well as surface α-glucan content. Elevation in the level of glycogen was observed for yeast cells grown in YPD medium containing calcium. Calcium, a secondary messenger in eukaryotic cells plays an important role in glycogen metabolism enhancing the level of glycogen synthesis in yeast. Combined effect of $CaCl_2$ and ethanol also displays elevation in the level of surface α-glucan content in yeast cells (Table 1). These surface α-glucans play an important role in the process of flocculation in *Saccharomyces italicus*. This revealed the correlation between ethanol, calcium, insoluble glycogen representing α-β-glucan complex and flocculation in yeast. Apart from cell surface carbohydrate extracellular ethanol also affects surface glycoproteins in yeast cells. Action of ethanol on the cell wall of yeast results net increase in the level of surface glycoproteins like glucomannoproteins as well as galactomannoproteins.

Acknowledgements

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