Disruption of *GAL1* gene in *Saccharomyces cerevisiae* leads to higher ethanol production

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Abstract

GAL gene family is a set of structural and regulatory genes that enables cells to utilize galactose as a carbon source in Saccharomyces cerevisiae. Phosphorylation of intracellular galactose can be catalyzed by galactokinase (encoded by GAL1 gene). In this study role of GAL1 gene on ethanol production by S. cerevisiae together with physiological characterization of GAL1 mutant strain was studied. Aerobic cultivation was carried out with wild-type strain and the GAL1 mutant. The GAL1 mutant strain displayed fermentative growth in early exponential phase. Deletion of the GAL1 gene was shown to have a major impact on biomass and ethanol formation. The GAL1 mutant exhibited a decrease in growth rate and increased ethanol production. Furthermore the results showed that glucose consumption by GAL1 mutants did not favor biomass formation, rather cause excessive respiro-fermentative metabolism, with whereas could linear increase in ethanol production.

Keywords: *GAL1, Saccharomyces cerevisiae, Gene disruption, Ethanol.*

INTRODUCTION

Ethanol is one of the most important products originating from the biotechnological industry with respect to both value and amount. Two-third of the production is located in Brazil and United States with the primary objective of using ethanol as a renewable source of liquid fuels. This market is expected to result in a substantial growth in the ethanol production industry in the near future. There are therefore strong economic incentives to further improve the ethanol production process. The price of the sugar source is a very important process parameter in determining the overall economy of ethanol production, and it is of great interest to optimize the ethanol yield in order to ensure an efficient utilization of the carbon source (Nissen *et al.,* 2000).

The yeast *Saccharomyces cerevisiae* utilizes a variety of carbon sources for growth, but glucose and related hexoses are used preferentially. Inactivation of structural and regulatory genes by disruption or replacement has become a matter tool for physiological studies (Davis *et al.,* 1998). By comparing mutants with the wild type, important information can be obtained about the physiological role of the gene involved (Ostergaard *et al.,* 2000). The GAL system, which contains the genes that encode the proteins responsible for galactose utilization, is subjugated to dual control being induced by galactose and repressed by glucose (Oh and Hopper, 1990). The yeast *GAL* genes have provided a widely used model for studies of eukaryotic gene regulation (Lohr *et al*., 1995). The galactose utilization pathway i.e., the leloir pathway, responsible for catabolism of galactose to glu-6P, involves several enzymatic reactions (Johnston *et al.,* 1994). Galactose permease encoded by the *GAL2* gene is then phosphorylated at position 1 under consumption of ATP by galactokinase encoded by the *GAL1* gene (Horak and Wolf, 1997). *GAL7* encodes a galactose-1-phosphate uridylyltransferase that uses *GAL1*-1P and UDP-glucose as substrates for the formation of GLU-1P and UDP-galactose. Later component is then

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reconverted into UDP-glucose by action of UDP-glucose 4-epimerase encoded by the *GAL10* gene. Finally, the phosphate group of glu-1p is transferred to position 6 by phosphoglucomutase encoded by the *GAL5* gene synonymous with the two isoenzymes encoded by *PGM1* and *PGM2* (Dejuan and Lagunas, 1986). Control of carbohydrate metabolism in *S. cerevisiae* and other yeasts is of both fundamental and practical significance and has been the subject of several studies over the past decade (Ostergaard *et al.,* 2000; Alfenore *et al.,* 2002; Johnston *et al.,* 1994; Horak and Wolf, 1997 and Shi *et al.,* 1999). *S. cerevisiae* displays the Crabtree effect: When it grows on glucose under aerobic conditions, the sugar is largely fermented to ethanol rather than respired. This effect is due in part to the repression at high glucose concentrations of genes encoding respiratory activities. Glucose repression is a complex regulatory system controlling numerous biochemical pathways (Klein *et al., 1998*). In this study, we investigated the role of *GAL1* in ethanol production by comparing ethanol production and biomass of the wild-type strain and GAL1 mutants in glucose substrate.

MATERIALS AND METHODS

Microorganisms: Two *S. cerevisiae* strains were used throughout this study: *S. cerevisiae* 2805 (*MATa pep4::HIS prb-Δl.6R canl his3-20 ura3-52*) and the GAL1 mutant.

Culture growth and preservation: Shake-flask cultures of the strains were grown on YPD medium (Difco yeast extract, 10 g/l, Difco peptone, 10 g/l, glucose, 20 g/l). Sterile glycerol was added to the overnight cultures to give a final concentration of 15% (v/v). One milliliter stock vials were stored at -80°C as main working stock and each vial was used for inoculation of precultures for growth experiments.

***GAL1* gene disruption:** The *GAL1* gene was replaced by a *URA3* cassette in the wild-type strain to produce the GAL1 mutant strain as outlined in figure 1: N and C terminal fragments were amplified from wild type by polymerase chain reaction (PCR). *Gal1*F1(GACGAATTCAATTGGCAGTAACCTGG) and *Gal1*R1 (GCAGGATCCGAACTCAGGTACAATCAC) (underlined nucleotides correspond to the *Eco*RI and *Bam*HI sites, respectively) are the upstream and downstream primers, respectively, used for the amplification of N terminal fragment. *Gal1*F2 (CGCGGATCCGTTCTTGTCCAGAGATTGAC) and *Gal1*R2(CGCTCTAGAGACAGCT GCC CAATGCTGG) (underlined nucleotides correspond to the *Bam*HI and *Xba*I sites, respectively) are the upstream and downstream primers, respectively, used for the amplification of C terminal fragment. The PCR amplified fragment containing the N and C terminal were digested with *Eco*RI, *Bam*HI and *Bam*HI, *Xba*I respectively. pBLuscript II KS was digested with *Eco*RI and *Xba*I. The fragments were isolated in %8 agarose gel and then ligated to the vector. The ligation mixture was transformed into *E. coli* to give pBSGal1NC. A 2 kb *Bam*HI fragment containing TC5::*URA3* cassette was inserted into pBSGalNC at the *Bam*HI site, creating pBSGal-NCURA3. The N::*URA3*::C cassette fragment was removed from pBSGalNC*URA3* with *Eco*RI and *Xba*I and transformed into wild-type by the lithium acetate method (Gietz et al., 1992 and Hill et al., 1991). Transformants were grown on SD medium *URA*- derivatives of transformants were initially obtained by patching onto 5-*FOA* plates, which are selective for *URA3* strain (Pronk *et al.,* 1996; Alani *et al.,* 1987 and Davis *et al.,* 1998).

Media and culture conditions: In shake-flask cultures, triple-baffled 250-ml shake flasks were used to ensure adequate mixing and oxygen transfer. Duplicate flasks containing 25 ml growth medium in YPD (1% yeast extract, 2% peptone, 2% glucose) were inoculated with pre-culture cells in YPD. The culture was conducted with vigorous shaking at 30°C.

Cell mass determination: The cell mass concentration on a dry weight basis was determined by spectrophotometric measurements at 620 nm in a spectrophotometer (Hitachi U-1100) and calibrated against cell dry weight measurements. The spectrophotometric measurements enabled evaluation of doubling of the population during fermentation. Cells were harvested by filtration on nitrocellulose filters with a pore size of 0.45 μm. Initially the filters were predried in a microwave oven at 150 W for 10 min, and weighed. A known volume of cell culture was filtered and the residue was washed with distilled water. Finally, the filter was dried in the microwave at 150 W for 15 min, and weighed (Rose *et al., 1990*).

Analysis of extracellular metabolites: For determination of the extracellular metabolites, 1.8 ml of sample was taken out of the flask and immediately filtered through a 0.45 μm pore size cellulose acetate filter (Sartorious AG, Gottingen, Germany). The glucose,

galactose and ethanol were separated on an Aminex HPX-87H column (Biorad, Hercules, CA) at 50°C using 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 ml/min. Glucose, galactose and ethanol were detected refractometrically (Waters 410 differential refractometer detector; Millipore Corp, Milford, M.A) (Ostergaard *et al.,* 2000).

RESULTS

GAL1 mutant strain was constructed *in vitro* as described above. The resulting strain was designated *S. cerevisiae* 2807 (*MATa pep4::HIS prb-Δl.6R canl his3-20 ura3-52* Δ*Gal*). Figure 1 shows that pBGal1 (N/C/UR3) was constructed, which contain the *Gal1*N-C gene on *Xba*I- *Eco*RI fragment, as well as the insertion mutation (the Ura3 fragment into the *Bam*HI site). The insertion mutation in the *Gal1* gene was then introduced onto chromosome. The mutations were introduced back into the yeast genome by integration directed at the *Gal1* locus followed by excision of the plasmid and loss of the wild type allele. This was achieved by homologous recombination. The aerobic physiology of the genetically engineered *S. cerevisiae* was studied in aerobic batch cultivation using glucose as carbon source. This was done to quantify the effect of the genetic changes on the ethanol production. We measured growth rate, glucose consumption, and ethanol production by the *Gal1* mutant and wild type at 2% glucose concentration. The biomass concentrations during the exponential growth phases

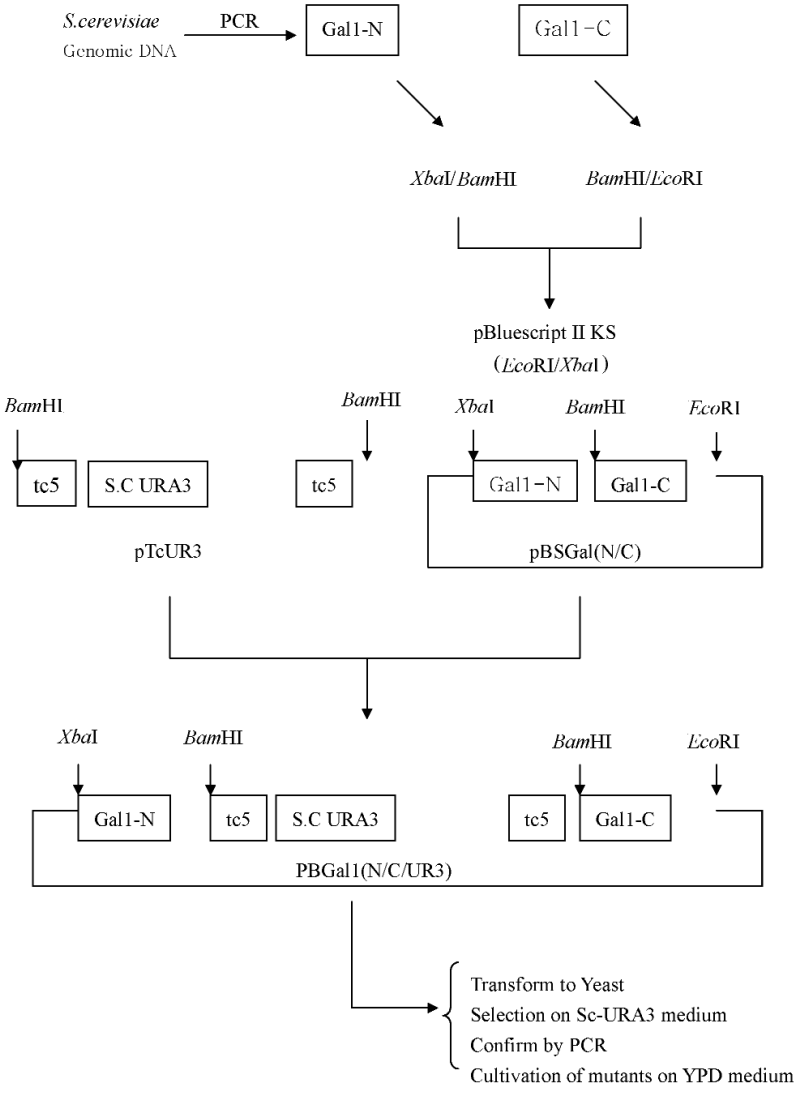

Figure 1.*Gal*1 gene disruption in *Saccharomycess cerevisiae.*

of wild type and GAL1 mutant are depicted as functions of time. Deletion of GAL1 resulted in a reduction in the growth rate (Fig. 2). The exponential growth rates of the wild type were decreased with mutation in *Gal1* gene. The growth rate of the wild type decreased from 0.31 h^{-1} at 2% initial glucose to 0.26 h^{-1} on Gal1 mutant strain. The consumption of glucose was measured in filtered samples withdrawn from the batch cultivations of wild type and Δ*GAL1* strains (Fig. 3). Production of ethanol as function of time was shown in cultivations of the strains (Fig. 4). The best performance was found at 30°C around 1.2 gl^{-1} ethanol produced in 120h. In all cultivations the ethanol production was stopped immediately after depletion of glucose in the medium. The ethanol yield increased significantly in cultivations of GAL1 mutants compared with wild type while the growth and biomass production were reduced. The ethanol productivity was significantly affected following gene deletion because the biomass in Δ*GAL1* mutant was approximately half of that in the wild type.

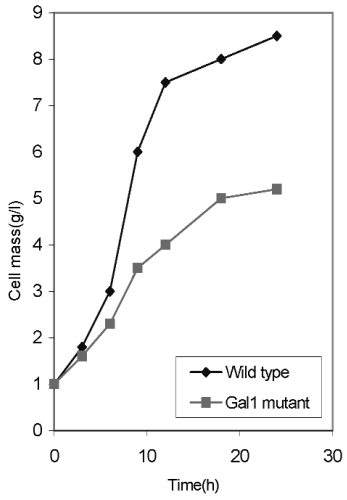

Figure 2. Effect of *Gal1* gene in growth. Wild type and *Gal1* mutant were grown on YPD.

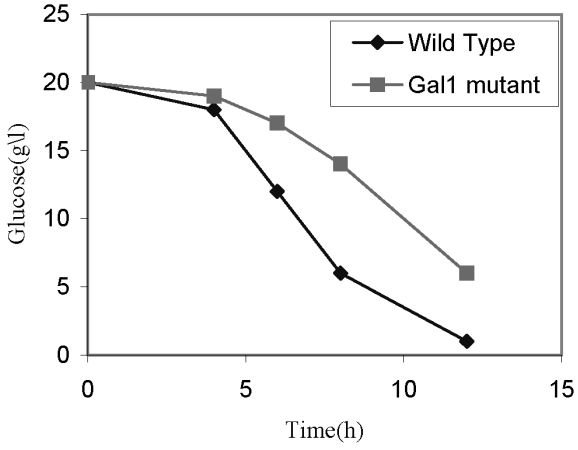

Figure 3. Effect of *Gal1* mutant on glucose consumption.

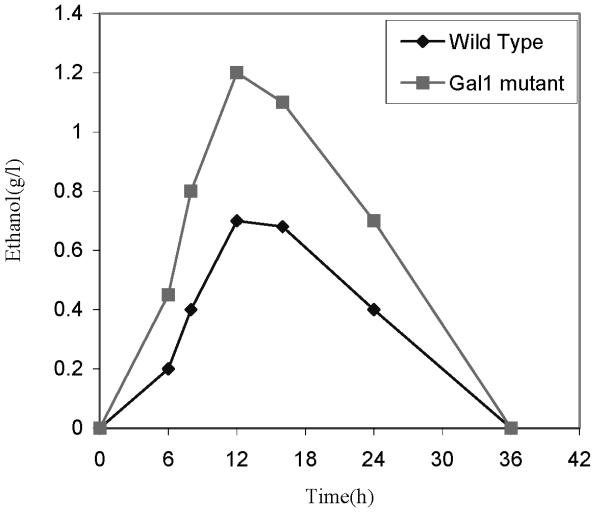

Figure 4. Ethanol production of wild type and *Gal1* mutant.

DISCUSSION

Galactose can be utilized as the nutrient by *S. cerevisiae*. Mutation in *GAL1* gene was constructed *in vitro* as described above. In order to investigate the impact of *GAL1* disruption in the wild type background on glucose metabolism, strains were grown in aerobic batch cultivations on YPD medium. Deletion of *GAL1* resulted in reduction of the growth rate (Fig. 2). This was probably due to an increase in the rate of ethanol synthesis (Klein *et al.,* 1999). Usually, sugar concentrations above 20% (W/V) are not used under industrial conditions because increasing the concentration of ethanol delays the growth of the yeast (Alfenore *et al.,* 2002). Biochemical pathways in yeasts may be regulated at various levels. These include: enzyme synthesis (e.g. induction, repression and derepression of gene expression), enzyme activity (e.g. allosteric activation, inhibition or interconversion of isoenzymes) and cellular compartmentalization e.g. mitochondrial localization of respiratory enzymes (Johnston and Carlson, 1992). Yeast, in particular *S. cerevisiae*, is used in many different biotechnological processes (Geoffrey and Cregg, 1999). These can be broadly divided into two categories depending on the metabolic state of the yeast cell. Production of ethanol occurs under fermentative growth conditions. Under respiratory growth conditions, yeast biomass is produced commercially in large scale fermentation processes. *S. cerevisiae* cul-

tures are increasingly used for the production of recombinant proteins or other modern biotechnological substances. Production of sufficient amounts of *S. cerevisiae* biomass stands also central to, and often initiates or is prerequisite for all fermentative yeast processes and also for making enough yeast available to satisfactorily start a fermentative process. The two different process categories depend on the presence of a fermentative and a respiratory route for the metabolism of fermentable substrate for energy production. Yeast is able to adapt its cellular composition towards each mode of growth, making it a versatile, but sometimes intractable organism. (These modes differ considerably in the yield of biomass on substrate). For the application of yeast often metabolic characteristics are required, that are normally obtained during fermentative growth conditions. Both modes need to be dealt with satisfactorily to reach to optimized production (Geoffrey and Cregg, 1999). A fermentative mode of growth is obtained under anaerobic conditions, in which the metabolic balance dose not allows the complete oxidation of the substrate without the supply of oxygen (Verduyn *et al.,* 1990). Also, many yeast strains show at aerobic conditions fermentative activity parallel to respiratory metabolism. Several authors have observed that yeast extract, ammonium, magnesium, calcium have a protective effect either on growth, fermentation, or viability, which overall stimulate the rate of ethanol production (Alfenore *et al.,* 2002; Flores *et al.,* 2000; Johnston and Carlson, 1992). In *S. cerevisiae,* by modifying nutritional conditions, it is possible to increase ethanol production by traditional fed-batch (Panchal and Stewart, 1981). High ethanol production in fermentation using *Saccharomyces cerevisiae* are determined by several factors such as medium composition and operating parameters including substrate and vitamin feeding strategy, oxygen level and temperature (Aldiguier *et al.,* 2004). Higher ethanol production was reported after disruption of cytochrome gene in yeast (Shi *et al.,* 1999).The impact of ethanol and temperature on the dynamic behaviour of *S. cerevisiae* in ethanol biofuel production was studied using isothermal fed-batch process at five different levels 27, 30, 33, 36 and 39°C (Aldiguier *et al.,* 2004). The ethanol yield increased significantly in cultivations of GAL1 mutant compared with wild type while the growth and biomass production were reduced. This was probably due to formation of some byproducts (Alfenore *et al.,* 2002).

In *S. cerevisiae*, a large number of genes are turned off during growth on glucose. These glucose repressible genes can be divided into three groups:

I) Genes for metabolizing other carbon sources
II) Genes encoding enzymes for gluconeogensis
III) Genes involved in the Kerebs cycle and in respiration

In the first exponential growth phase, the glucose is metabolized predominantly to ethanol and CO_2, with the minor products of fermentation being glycerol, acetate, and pyrovate. Genes for metabolizing carbon source are important in production of some bioproducts. In yeast, glucose acts as a source of free energy. Glucose is oxidized to ethanol (fermentative catabolism) or CO_2 (oxidative catabolism), and this is coupled with ATP synthesis via substrate level phosphorylation or oxidative phosphorylation. The branch between fermentative and oxidative growth occurs at the level of pyruvate. This metabolite is formed by glycolysis, and can either be converted to ethanol via pyruvate decarboxylase and alcohol dehydrogenase, or oxidized via the pyruvate dehydrogenase complex and tricarboxylic acid cycle. Some of intermediary metabolites are substrates for biosynthesis. Pyruvate is a precursor for the synthesis of branched chain amino acids and CoA (Pronk *et al.,* 1996).

The galactose utilization pathway i.e., the Leloir pathway, responsible for catabolism of galactose to Glu-6P, involves in several enzymatic reactions. Our results show the galactose pathway can have some effect on ethanol production. It has been shown that regulation in a metabolic network can be controlled by some byproducts (Pronk *et al.,* 1996; Johnston and Carlson, 1992). A number of byproducts are found during growth *S. cerevisiae*. Glycerol is one of the most important of byproducts, produced by yeast to reoxidize NADH, formed in synthesis of biomass and secondary fermantation products. Glycerol formation has two physiological roles in *S. cerevisiae.* Synthesis of biomass and organic acids, i.e., succinic acid, acetic acid, and pyruvic acid, results in a net formation of intracellular NADH. This must be balanced by a mechanism in which NADH is reoxidized to NAD^+ in order to avoid a serious imbalance in the NAD^+/NADH ratio. Under anaerobic conditions the respiratory chain is not functioning. Instead, NADH is reoxidized to NAD^+ by formation of glycerol, and synthesis of 1 mol of glycerol from glucose leads to reoxidation of 1 mol of NADH (Flores *et al.,* 2000).

Deletion of *GAL1* in *S. cerevisiae* resulted in an increase in ethanol formation and a decrease in biomass. The controlled genes can be divided into peripheral functions, i.e. metabolism of saccharides to glucose-6-phosphate as well as further influx/ efflux functions, and central functions, such as gluconeogenesis, the glyoxylate shunt, fermentative functions, the tri-

carboxylic acid cyclic acid and oxidative phosphorylation. *GAL1* deletion has a greater impact on peripheral functions, pyruvate, than on central metabolism. During aerobic batch growth on 2% glucose, distinct growth phases could be distinguished in wild type *S. cerevisiae*. As a conclusion, our studies have shown that the disruption of *GAL1* improves a industrially relevant physiological characteristics of *S. cerevisiae*, higher ethanol production. The characteristics contributes to a better economy of industrial yeast processes.

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