MIG1 Glucose Repression in Metabolic Processes of Saccharomyces cerevisiae: Genetics to Metabolic Engineering

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Abstract

Background: Although Saccharomyces cerevisiae has several industrial applications, there are still fundamental problems associated with sequential use of carbon sources. As such, glucose repression effect can direct metabolism of yeast to preferably anaerobic conditions. This leads to higher ethanol production and less efficient production of recombinant products. The general glucose repression system is constituted by MIG1, TUP1 and SSN6 factors. The role of MIG1 is known in glucose repression but the evaluation of effects on aerobic/anaerobic metabolism by deletion of MIG1 and constructing an optimal strain brand remains unclear and an objective to be explored.

Methods: To find the impact of MIG1 in induction of glucose-repression, the MIG1 disruptant strain (ΔMIG1) was produced for comparing with its congenic wild-type strain (2805). The analysis approached for changes in the rate of glucose consumption, biomass yield, cell protein contents, ethanol and intermediate metabolites production. The MIG1 disruptant strain exhibited 25% glucose utilization, 12% biomass growth rate and 22% protein content over the wild type. The shift to respiratory pathway has been demonstrated by 122.86 and 40% increase of glycerol and pyruvate production, respectively as oxidative metabolites, while the reduction of fermentative metabolites such as acetate 35.48 and ethanol 24%.

Results: Results suggest that ΔMIG1 compared to the wild-type strain can significantly present less effects of glucose repression.

Conclusion: The constructed strain has more efficient growth in aerobic cultivations and it can be a potential host for biotechnological recombinant yields and industrial interests.

Keywords: Saccharomyces cerevisiae, Homologous recombination, Metabolic pathways, Yeasts
The secreted form of invertase is glycosylated and located in periplasmic space, digesting extracellular sucrose to glucose and fructose, both of which can be transported into the cell. The cytoplasmic form is not glycosylated, and acts physiologically to cleave sucrose, transported across the plasma membrane.

SUC2 gene repression following glucose uptake constitutes a regulatory cascade through a large number of regulatory elements. Miglp, a zinc finger DNA-binding protein, is the main involved component that mediates glucose induced repression of sugars. Inactivation of MIG1 gene derepresses the expression of invertase encoded by SUC2. This protein resembles the mammalian Egr and Wilms’ tumor proteins, the CreA repressor in Aspergillus nidulans and the Migl repressor in Kluyveromyces lactis.

The MIG1 promoter has been reported to be auto-regulated. Miglp prevents transcription of SUC2 through the mechanism that mediates the binding of the general repressor Ssn6p-Tup1p complex to the regulatory part of SUC2. Snf1 kinase regulates glucose derepression of genes required for utilization of alternative carbon sources. The main mechanism is through glucose dependent dephosphorylation of transcriptional repressor protein Migl. One important strategy to reveal the mechanism of Mig1-mediated repression is to investigate the physiological consequences of MIG1 deletion/disruption and/or MIG1 over expression. No absolute evidence could well explain the physiological changes by Migl-redundant protein in a ΔMIG1 mutant, i.e. a protein that is similar to Miglp and can partly be a substitute. A second repressor, Mig2p, which is 71% identical to Miglp, as well as a related protein, YerO28, have been identified. In microarray approach combinational control, MIG1 and MIG2 repress a largely overlapping set of genes but MIG3 does not seem to overlap in function with MIG1 and MIG2. Instead, MIG3 down regulates the SIR2 gene responsible for gene silencing and the control of aging. There are two approaches to silence MIG1 gene of which one is usually accomplished by disruption and substitution of the target gene with a selecting marker introduced by homologous recombination. The second alternative approach, which has been applied and researched in plant, is antisense gene expression.

In this study, the purpose was to apply the first approach to disrupt chromosomal MIG1 gene through homologous recombination with reconstructed plasmid containing inserted N and C terminals of MIG1 gene coupled with URA3 selecting marker enabling ΔMIG1 mutants to be screened. Disruption and replacement of MIG1 can derepress promoters of genes which are repressed by glucose. Prototrophic MIG1 disruptant (∆MIG1) as well as its congenic wild-type strain (2805) were analyzed for expected physiological changes in peripheral metabolism (batch cultivations on sugar mixtures) and central metabolism. During the cultivations, an attempt was made to monitor the parameters of glucose consumption, biomass production, intracellular protein content and patterns of metabolite production to understand how the effect of MIG1 removal can shift to oxidative pathway of metabolism. The strain can be optimized as a host for further processing of recombinant production with advantages of being eukaryotic.

Materials and Methods

Preparation of YPD broth culture to grow the yeast

S. cerevisiae wild type strain 2805 was cultured on solid or liquid Yeast Peptone Dextrose (YPD), while carbohydrates were supplied at concentrations of 20 g/L. For physiological experiments, a medium, previously described, was used with minor modifications. Medium for batch cultivations consisting of glucose (28 mM), sucrose (58 mM), (NH₄)₂SO₄ (7.5 g/L), KH₂PO₄ (3.5 g/L), MgSO₄·7H₂O (2.7 g/L), trace metal solution (20 mL/L), vitamin solution (2 mL/L), and antifoam 289 (50 mL/L) were used. The trace metal solution was composed of EDTA (3 g/L), CaCl₂·2H₂O (0.9 g/L), ZnSO₄·7H₂O (0.9 g/L), FeSO₄·7H₂O (0.6 g/L), H₂BO₃ (0.2 g/L), MnCl₂·2H₂O (155.5 mg/L), Na₂MoO₄·2H₂O (80 mg/L), CoCl₂·2H₂O (60 mg/L), CuSO₄·6H₂O (60 mg/L), and KI (20 mg/L), adjusted to pH=4.0 with NaOH. The vitamin solution was composed of d-biotin (50 mg/L), p-aminobenzoic acid (200 mg/L), nicotinic acid (1.0 g/L), calcium pantothenate (1.0 g/L), pyridoxine HCl (1.0 g/L), thiamine, HCl (1.0 g/L), and m-inositol (25 g/L), adjusted to pH=6.5 with HCl. Sugars were autoclaved separately, and the vitamin solution was sterilized by filtration and added after autoclaving. To prevent bacterial growth antibiotics penicillin G, streptomycin and chloramphenicol were added to media in concentration 100, 100 and 50 µg/mL, respectively along with adding 2% agar which can provide YPD solid media. The preparation of the inoculum and the conditions of batch cultivation were according to the described experiment: a colony taken from YPD agar plate inoculated in 4 baffled Erlenmeyer shake flasks containing 250 mL YPD broth at 28°C and 100 rpm for 48 hr. The temperature was kept fixed and the pH was controlled within the range 4.9-5.0 by automatic addition of 4 M NaOH. The air flow was set to 1vvm (4.0 L/min), and the off-gas was led through a condenser. After incubation period, media were centrifuged and washed by normal saline within 50 mL tubes in 3000 rpm for 10 min.

Preparation and analysis of DNA

Total yeast DNA was extracted as described by Ausubel et al. To check purification of extracted DNA, quantitative control was performed by spectrophotometric method and quality of DNA was checked by accuracy and sharpness of related bands in gel electrophoresis. For restriction analysis and gel electrophoresis, experiments standard protocols were followed. For the purification of DNA fragments used for cloning experiments, the desired fragments were separated on
0.7% agarose gels, excised and recovered from agarose, using BIOTRAP BT1000 (Schleicher & Schuell, Duren, Germany).

Oligonucleotide primers and PCR analysis
Both forward and reverse primers provided by Oligo and Blast software for each of N-fragment (before starting codon of MIG1) and C-fragment (after stop codon of MIG1) included restriction sites for EcoRI and BamHI. Primers were made by Clontech Company according to the N- and C-terminal sequences of MIG1 gene. PCR amplification of N and C terminal fragments was performed by thermocycler according to instructions followed: initial single denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 45°C for 45 s, extension at 72°C for 1 min and final extension at 72°C for 5 min. The correct amplification of PCR products (N-fragment of 400 bp, C-fragment of 600 bp) was confirmed by gel electrophoresis. In table 1, the oligonucleotide primers used in PCR analysis are shown.

Cloning of N and C fragments and transformation to Escherichia coli (E. coli)
The amplified N and C fragments were cleaned up by PCR product purification kit (Biogen Company). The purified products enabled restricted digestion of fragments on suitable restriction sites which existed within the primers. Namely, N fragments were digested with EcoRI and BamHI enzymes and C fragments were digested with XbaI and BamHI enzymes. The restriction digestion reactions were carried out for overnight at 37°C. The enzymes were inactivated in 10 min at 65°C. After gel electrophoresis of digested fragments, 400 and 600 bp DNA bands corresponding to N- and C-fragments, respectively, were excised from gel. The gel purified N- and C-fragments were inserted into plasmid pBluescript II SK (Stratagene) using Fermentase DNA cloning kit (Thermo Fisher Scientific Company). The resulted constructed plasmid pMIG1 consists of BamHI as midline restriction site between inserted N- and C-fragments. Furthermore, plasmids were transferred to Escherichia coli (E. coli) DH5a (Bethesda Research Laboratories). E. coli was transformed by electroporation using a Gene Pulser (Pulse Controller, Bio-Rad, Richmond, CA, USA) according to the manufacturer’s instructions. The growth of transformants in LB culture media containing ampicillin (100 mg/ml) and X-a-gal (as screening agent) and IPTG (as galactose metabolism inducing factor) allowed selection of white colonies consisting of pMIG1 plasmid. The recombinant pMIG1 plasmid consisted of plasmid pBluescript, N- and C-fragments digested with BamHI and inserted with selection marker, URA3, as described by Klein et al. 17.

Transformation to yeast by pMIG1 plasmid
The wild strain S. cerevisiae was transformed by PEG/Li acetate method 16 to replace to produce MIG1 disrupted strains. URA3 enables recombinant yeast to synthesize required uracil for growing in uracil-lacked medium (SC-URA).

Determining cell biomass
Produced biomass of wild and MIG1 disrupted strains was determined by using optical densitometry method. The biomass yield was obtained from the slopes of the plots of biomass and the amount of consumed sugar during exponential growth phase.

Analysis of sugars and produced metabolites
To measure sugars and extracellular metabolites, cultivation medium was sampled, immediately filtered through a 0.45 mm pore size cellulose acetate filter. Glucose, galactose, maltose, ethanol, glycerol, acetate and pyruvate were separated on an Aminex HPX-87H ion exclusion column HPLC and were detected refractometrically or spectrophotometrically 13.

Measurement of cell protein content
For the determination of cell protein content, a sample was isolated in ice beaker, centrifuged and washed three times with 5 ml of 0.9% (w/v) NaCl and resuspended in 1 ml of water, before storage at -80°C. Then, the total protein content was measured according to the Biuret method 12.

Results
Construction of disrupted mutant yeast
The haploid wild type strain 2805 was selected to test the feasibility of suppressing effect of MIG1 by gene disruption and to simplify direct isogenic comparison of MIG1 expressing strains with that of MIG1 disrupted mutants. The confirmation of amplification of N and C fragments of MIG1 gene sequence with 400 and 600 bp, respectively, were compared to size marker (Figure 1). Insertion of N and C fragments in pBluescript II plasmid and transformation to E. coli DH5a leading to antibiotic resistance attributed to the bacterial aminoglycoside phosphotransferase-encoding APT2 gene which is inserted between the PGK promoter and terminator.

After replica plating to X-a-Gal medium under inducing conditions with galactose and glycerol, transformed bacterial strains were isolated (data not shown). Upon transformation of strain 2805 with pBluescript II integrated with N and C fragments of MIG1 sequence interrupted with URA3, several clones of yeast able to grow in SC-URA were isolated.

Metabolic assessment
For batch cultivations on glucose, each of the Saccharomyces strains 2805 and ΔMIG1 were grown on

Table 1. Oligonucleotide primers used in PCR analysis

| PRIMER | ENZYME | RESTRICTION SITE |
|--------|--------|------------------|
| F1     | XbaI   | CCCCTCTAGAGAATTACGAGCCATAG |
| R1     | BamHI  | CCCGGGATCTGCGAGTTTATATTAGT |
| F2     | BamHI  | CCCCCGGATCTGCGGCTTCTACTATAT |
| R2     | EcoRI  | CCCGAATTCCAACTTTGAGCATCCTGAA |

Avcienca Journal of Medical Biotechnology, Vol. 11, No. 3 July-September 2019
Ethanol production and cell protein content demonstration 0.39 and 0.43 calculated from nine simultaneous maximum specific growth rate of 2805 and of ∆MIG1 chromosomal gene of Saccharomyces Cerevisiae next to the size marker. The size marker (SM) contains of bands for each equals to 100 bp.

Glucose consumption and growth rate

Glucose consumption in ΔMIG1 mutant was proceeded to wild 2805 strain by 25.0% within several batch and lag phase measurements (Figure 2A). The maximum specific growth rate of 2805 and of ΔMIG1, calculated from nine simultaneous optical density measurements demonstrated 0.39 and 0.43 h⁻¹, respectively. Thus, MIG1 disruption led to 12.0% average increase of specific growth rate on glucose (Figure 2B).

Ethanol production and cell protein content

In batch cultivation, the rate of carbon flux directed to ethanol production in ΔMIG1 strain was reduced about 24.0% (C-mmol/L) of lag phase cultivation (Figure 2C). The less formation of ethanol and related anaerobic metabolites at a higher rate with ΔMIG1 than with 2805 demonstrates considerable shifted metabolism from respiro-fermentative to respiratory pathway. Intracellular protein content accounted for both strains showed 22.0% (w/w) difference to significant increase in ΔMIG1 compared with 2805 (Figure 2D).

Glycerol, pyruvate and acetate formation

The carbon fluxes have been increased to respiratory metabolism pathway. It is indicated by more remarkable formation of oxidative metabolites markers, i.e. glycerol and pyruvate which have been produced 122.86 (C-mmol/L) and 40.0% (C-mmol/L) in ΔMIG1 prominent than strain 2805, respectively. Conversely, the decrease of fermentative metabolite of acetate by 34.58% (C-mmol/L) has been observed. The differences between the rate of metabolite production within cultivation period have been calculated and expressed as percentage (Δ%) (Figure 3). The comparison between all continuous variables was performed by using one-way analysis of variance (ANOVA). If there were statistically significant differences, then Tukey’s Post-Hoc test was applied. The values of p<0.05 were considered statistically significant.

Discussion

Yeast has been considered as a perfect microorganism for biological studies and protein recombinant production. Safety of handling and synthesized recombinant products also capable genetic transfer systems make this organism to be widely used as a host. In yeast cells, unification of transformed DNA and chromosomes potentially occur through homologous recombination process leading to expression of different modified eukaryotic proteins.

The major types of yeast being used in biotechnology as gene expression tools are S. cerevisiae, Hansenula polymorpha, and Kluyveromyces lactis. For several
advantages, *S. cerevisiae* is well selected for heterolo-
gous protein production. In this research, the chromo-
somal metabolic gene, *MIG1*, disrupted by homologous
recombination through antisense technique using up-
ward and downward, N- and C- fragments of *MIG1*
gene was applied to study the metabolic effects on
yeast. Lateral fragments, N and C, after amplification
were inserted in yeast specified vector, pTcURA3
alongside metabolic selection marker URA3. After
transformation to yeast, *MIG1* gene would be replaced
with URA3 marker. It can be happened by recombi-
ation between its lateral N and C fragments and resem-
bles sequences already inserted into the vector lateral
to URA3 marker. This transformation enables the yeast
to synthesize required nutrient uracil. In fact, through
this method, *MIG1* disrupted strain can be screened by
growth on uracil negative medium. Then, further anal-
yses of central and peripheral metabolic pathway have
been done to approach variations between mutant and
intact wild strain.

The glucose accessibility is the most important envi-
nmental factor to regulate oxidation and fermentation
metabolic pathways of *Saccharomyces i.e.*, glucose
concentration in batch inhibits the utilization of other
carbon resources, the catabolic inhibition 19. Hexoki-
nase PII starts glucose inhibition cascade 19 and Snf1p
as a signal transductor phosphorylates miglp (regulato-
ry zinc finger protein of *MIG1* gene) which makes inhi-
bitory complex with Ssn6 (cyce8)-tup1 components
(Carlson, 1987) and binds to promoter of glucose con-
trolled genes. The *MIG1* gene was cloned in 1990 for
the first time 4. Deletion of *MIG1* demonstrates a grea-
ter impact on peripheral functions than on central me-
tabolism.

There are recent evidences which persist on the role
of Hexokinase 2 (Hxk2) in regulation of DNA-binding
repressor proteins of Mig1 protein and glucose repression
signal in nucleus. Hxk2 acts as an intracellular
glucose sensor that operates by changing its confor-
mation in response to cytoplasmic glucose levels and
regulates dephosphorylation of Mig1 19,20. In the pre-
sent study, ∆*MIG1* strain 2805 could utilize more glu-
cose in comparison to the wild strain within the first 12
hr to reach to the zero point. When the glucose con-
centration is high, Snf1p is inactivated and non-phos-
phorylated miglp would remain in nucleus and represses
transcription of dependent genes. Conversely, in low
concentration of cultivation glucose, phosphorylated
MIG1p can migrate to cytoplasm and leads to removal
of repression status. The deletion of *MIG1* can elimi-
nate glucose repression partially from peripheral func-
tions, rather than completely from central metabolic
functions. The outcome of this deletion has been con-
sidered significant in industry because glucose is the
most useful sugar among the carbon resources for yeast
in industry process. This can delay uptake of other sug-
ars resulting in elongation of production process.
Therefore, an effective alleviation of glucose control
would help to achieve a better process economy for the
cultivation of baker’s yeast, alcohol fermentation,
bread-making and recombinant protein production 17.
In some studies, it has been proved that disruption of
*MIG1* cannot remove the entire glucose repression ef-
fect from galactose, maltose and sucrose metabolism.
Therefore, substantial *MIG1*-independent glucose con-
trol mechanisms exist for the GAL, MAL and *SUC*
systems 21. On the other hand, glucose through *MIG1*
regulation process has several repression effects on the
expression of GAL family genes likeagal2, gal3, gal4,
and gal80. They are involved in the metabol-
ism of galactose and highly affected by *MIG1* disrupt-
ion 22,23. Imp2p is a *MIG1* related regulator of GAL
genes and has the positive effect on glucose derepres-
sion of the maltose, galactose and raffinose utilization
pathways and in resistance to thermal, oxidative or
osmotic stress of *S. cerevisiae* 23. The finding that
CAT8 was derepressed in a ∆MIG1 strain, whereas
Cat8-controlled FBPI, PCK1 and ZC1 were not, gives
a clear hint of the existence of other repressors such as
MIG2 or a post-translational modification of an effec-
tor such as the phosphorylation of the repressor Cat8
16.

The study of the effect of simultaneous deletion of
*MIG1/MIG2* genes on physiology of *Saccharomyces*
exhibited that glucose control of maltose and sucrose
metabolism was derepressed in disrupted *MIG1/MIG2*
3,24. However, the lag phase of galactose cultivation
diminished and further deletion of MIG2 could not
affect glucose dependent metabolism of galactose. The
*MIG1* gene was silenced by antisense *MIG1* expres-
sion 10. The results of evaluation of glucose consump-
tion, cell biomass production and intracellular protein con-
tents by Optical Densitometry (OD) method and In-
vertase enzymatic activity assays demonstrated the more
flexible growth of ∆*MIG1* strains under the suffi-
cient amount of oxygen and glucose. This is evidential
for removal of glucose repression effects and impres-
sive shift to more oxidative metabolism in comparison
to wild strains. Yeast cell cultivations are effectively
stressed by chemical metabolites produced within
growth phase namely acids and high percentage of eth-
anal which can suppress the yeast growth. Deletion of
*MIG1* can switch fermentative metabolism to aerobic
pathway. It results in the production of less fermenta-
tive products like ethanol and acetate. Meanwhile, the
promotion of Krebs cycle leads to more acidic deriva-
tive products such as glycerol and pyruvate. The data
from the current research approved the similar concept
12,25. However, the increase of glycerol production in
mutant strain is much prominent than what has been
reported in similar studies 10,13. Such modifications in
peripheral and central metabolic functions could satisfy
industrial interests which attempt to have optimal engi-
eered strains for recombinant production.
Conclusion

This study showed the amount of ethanol measured by HPLC method in mutant and wild strain cultivations which determined less production of $\Delta$MIG1. Enhancement of biomass production in $\Delta$MIG1 strains can lead to higher amount of intracellular protein content and carbohydrates. In the current research, results of strain $\Delta$MIG1 2805 could confirm similar achievements rather than wild types. As to peripheral functions, efforts have already been exerted in production of partially de-repressed galactose, maltose and sucrose metabolisms. For central functions, industrial interest could promote attempts in the metabolic engineering of strains that exhibit higher specific growth rates for baker’s yeast or recombinant protein production, or of distiller’s yeast strains that give higher ethanol yields.

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