International Journal of Food Microbiology 143 (2010) 150–160

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Increased biomass production of industrial bakers' yeasts by overexpression of *Hap4* gene

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ARTICLE INFO

Article history:
Received 16 April 2010
Received in revised form 19 July 2010
Accepted 11 August 2010

Keywords:
Industrial bakers' yeast
Biomass yield
Respiratory metabolism
Hap4p transcriptional activator
Invertase
Maltase
Lean and sweet doughs
Panification
Leavening capacity

ABSTRACT

HAP4 encodes a transcriptional activator of respiration-related genes and so, redirection from fermentation to respiration flux should give rise to an increase in biomass production in *Saccharomyces cerevisiae* transformants that overexpress *HAP4*. With this aim, three bakers' yeasts, that is, V1 used for lean doughs, its 2-deoxy-D-glucose resistant derivative DOG21, and V3 employed for sweet doughs, were transformed with integrative cassettes that carried *HAP4* gene under the control of constitutive promoter *pTEF2;* in addition VTH, DTH and 3TH transformants were selected and characterized. Transformants showed increased expression of *HAP4* and respiration-related genes such as *QCR7* and *QCR8* with regard to parental, and similar expression of *SUC2* and *MAL12;* these genes are relevant in bakers' industry. Invertase (Suc2p) and maltase (Mal12p) activities, growth and sugar consumption rates in laboratory (YPD) or industrial media (MAB) were also comparable in bakers' strains and their transformants, but VTH, DTH and 3TH increased their final biomass production by 9.5, 5.0 and 5.0% respectively as compared to their parentals in MAB. Furthermore, V1 and its transformant VTH had comparable capacity to ferment lean doughs (volume increase rate and final volume) while V3 and its transformant 3TH fermented sweet doughs in a similar manner. Therefore transformants possessed increased biomass yield and appropriate characteristics to be employed in bakers' industry because they lacked drug resistant markers and bacterial DNA, and were genetically stable.

1. Introduction

There are two main pathways for aerobic sugar metabolism in *S. cerevisiae* strains, namely, alcoholic fermentation and respiration (Gancedo, 1998, 2008; van den Brink et al., 2008). Fermentation prevails and substrate is mostly converted into ethanol in the presence of easily assimilable carbon sources such as glucose. The result is a lower biomass yield per glucose consumed than in respiratory metabolism (Franzen, 2003; Gancedo, 1998, 2008; Kummel et al., 2010; Turcotte et al., 2010). Alcoholic fermentation is highly desired in biotechnological processes such as elaboration of wine, brewing and baking, since it is necessary to generate metabolites during dough fermentation to give ethanol, carbon dioxide and proper flavors (Rehm et al., 1996). However, biomass production and high biomass yield is required prior to yeast application in industrial processes of metabolite conversion (Rehm et al., 1996; van Maris et al., 2001). Due to the low biomass yield obtained from fermenting cultures above a sugar concentration threshold, fermentation is avoided by substrate-limited fed-batch cultivation in biomass production industries such as bakers' yeast or production of enological yeast starters (Rehm et al., 1996).

Alcoholic fermentation that takes place even under aerobic conditions results from regulation exerted by the presence of glucose that activates several transcriptional regulators which, in turn, modulate expression of genes related to respiration, gluconeogenesis, utilization of carbon sources alternative to glucose, and others (Gancedo, 1998, 2008; Zaman et al., 2008).

Control of respiro-fermentative metabolism is conducted mainly by three transcriptional regulatory factors, i.e., Mig1p, Hap4p and Cat8p. Mig1p is the main catabolic repressor, whereas Hap4p and Cat8p activate respiration- and gluconeogenesis-related genes respectively (Kummel et al., 2010; Turcotte et al., 2010; Westholm et al., 2008). Mig1p binds to a 5′ sequence described in alternative carbon source metabolism-related gene promoters, gluconeogenesis-related genes and respiration-related genes such as *QCR8* and *HAP4* among others (Klein et al., 1998; Ostergaard et al., 2000; Rolland et al., 2002). Cat8p activates expression of gluconeogenesis-related genes and genes related to the glyoxylate cycle (Kummel et al., 2010; Ratnakumar et al., 2009; Tachibana et al., 2005; Turcotte et al., 2010). Hap4p, together with Hap2p, Hap3p and Hap5p transcriptionally activate the sequences UAS2 present, together with UAS1, in the promoters of respiratory-related genes, among others (McNabb and Pinto, 2005; McNabb et al., 1995). UAS1, UAS2 sequences and some regions of *HAP4* gene are highly

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doi:10.1016/j.ijfoodmicro.2010.08.009

Table 1
Yeast strains used in this study.

Strain	Relevant characteristic	Origen
S288C	Laboratory strain	YGSC[a]
STH*	S288C transformant that possesses the integrative cassette *ura3Δ*::p*TEF2*::ck*HAP4*::*CYC1*t	This study
BY4741	Laboratory strain *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF[b]
BYhap4Δ	BY4741 derivative that possesses the integrative cassette *hap4Δ*::KanMX4	EUROSCARF[b]
CEN. PK113-7D	Laboratory strain from which *HAP4* gene (ck*HAP4*) was amplified	P. Kötter[c]
V1	Bakers' yeast	CGL[d]
VTH*	V1 transformant that possesses the integrative cassette *ura3Δ*::p*TEF2*::ck*HAP4*::*CYC1*t	This study
DOG21	2-deoxy-D-glucose resistant mutant derived from V1	Rincon et al., 2001
DTH*	DOG21 transformant that possesses the integrative cassette *ura3Δ*::p*TEF2*::ck*HAP4*::*CYC1*t	This study
V3	Bakers' yeast	M. Tamame[e]
3TH*	V3 transformant that possesses the integrative cassette *ura3Δ*::p*TEF2*::ck*HAP4*::*CYC1*t	This study

[a] Yeast Genetic Stock Center, Berkeley, CA.
[b] European *Saccharomyces cerevisiae* archive for functional analysis.
[c] Johann Wofgang Goethe-Universität. Institut für Molekulare Biowissenchaften (Germany).
[d] Compañía General de Levadura, Valladolid (Spain).
[e] Instituto de Microbiología Bioquímica, CSIC, Salamanca (Spain).
* Transformants were named by three letters or numbers (STH, VTH, DTH, 3TH), that correspond to the first of their parentals (S288C, V1, DOG21, V3), the promoter (pTEF2) and the gene (HAP4).

conserved in other yeast and filamentous fungal species (Brakhage et al., 1999; Sybirna et al., 2005). *HAP2*, *HAP3* and *HAP5* genes are constitutively expressed, whereas *HAP4* is subjected to catabolite repression (DeRisi et al., 1997) by transcriptional regulation (McNabb and Pinto, 2005).

Isolation of *S. cerevisiae* strains with the metabolic flux partly diverted from alcoholic fermentation to respiration was feasible by either blocking the fermentative pathway, or by stimulating the respiratory pathway, or both (Blom et al., 2000; Lascaris et al., 2003, 2004; van Maris et al., 2001). Specifically the balance between respiratory and fermentative metabolism in *S. cerevisiae* laboratory strains was first diverted towards respiration by increasing expression of *HAP4* gene (Blom et al., 2000; Lascaris et al., 2003, 2004). Additional flaws in the glucose repression pathway such as deletion of *MIG1* or *HXK2* genes, Hxk2p being another component in the glucose repression pathway (Ahuatzi et al., 2004; Lascaris et al., 2004) increased the effect of *HAP4* overexpression (Kummel et al., 2010; Lascaris et al., 2004; Schuurmans et al., 2008; Turcotte et al., 2010) and contributed to more derepressed growth characteristics. However, *SUC2* expression was strongly reduced in the new strains, thus indicating that respiratory functions may repress transcription of genes related to alternative carbon source utilization such as sucrose (Lascaris et al., 2004; Schuurmans et al., 2008). That repression is most important for bakers' yeasts because sucrose, together with maltose, are present, first during bakers' yeast production (growth in molasses) and then during panification (lean and sweet doughs) (Rincon et al., 2001).

Overexpression of *HAP4* gene in industrial bakers' yeast strains was attempted by subjecting the gene to control of constitutive *TEF2* promoter *(pTEF2)*, which allowed to check whether or not respiro-fermentative metabolic regulation of industrial yeasts is similar to that of laboratory strains. In addition, it allowed the isolation of bakers' strains able to partly divert sugar metabolism to respiration, thus increasing biomass yield during bakers' yeast production.

2. Materials and methods

2.1. Strains

The *S. cerevisiae* strains used are described in Table 1. Laboratory strains S288C was selected as the laboratory control for transformations with an integrative cassette carrying *HAP4* gene under control of constitutive p*TEF2*, and STH transformant was characterized; CEN. PK113-7D, was chosen to amplify *HAP4* gene sequence; *HAP4* was deleted in BY4741 strain and its derivative mutant BYhap4Δ was transformed with the *HAP4* sequence from either CEN.PK113-7D or V1 strains to check functionality of gene. Bakers' strain V1, normally used for panification of lean doughs, was chosen for its high fermentative capacity (Codon and Benitez, 1995; Codon et al., 1995) as compared to other bakers' strains (Codon et al., 1997, 1998); DOG21 is a 2-deoxy-D-glucose resistant mutant isolated from V1 bakers' yeast that was chosen and characterized for its good qualities in bakers' industry (Codon et al., 2003; Rincon et al., 2001); V3 is a bakers' yeast commercially used to ferment sweet doughs. V1, DOG21 and V3 bakers' strains were transformed with the aforementioned integrative cassettes, and transformants carrying at least one copy of *HAP4* gene under the control of *pTEF2* (VTH, DTH and 3TH transformants respectively) were isolated and characterized.

Escherichia coli DH5α, commercialized by GE Healthcare (Buckinghamshire, UK), was used to propagate the different vectors used in this study.

Table 2
Plasmids/Vectors used in this study.

Name	Relevant characteristic	Origin
pGEMt-easy™	PCR cloning vector	Promega (Carlsbad, CA)
pUG6	Plasmid that contains the G418 resistant gene (KanMX4) flanked by *Lox*P direct repeat	Guldener et al., 1996
Yep351-Cre-cyh	Multicopy plasmid that contains the Cre recombinase gene under the *GAL1* promoter (p*GAL1*), *LEU2* and *CYH*^{R} genes	Delneri et al., 2000
pGDF1	Yep351-Cre-cyh derivative with the *LEU2* gene deleted after total digestion at *Hap*I and partial digestion at *Hin*dIII sites	F.A Guevara[a]
pRS416	Multicloning site (MCS) centromeric plasmid that contains *URA3* gene	Mumberg et al., 1995
p416tef	pRS416 derivative that carries *TEF2* gene promoter (p*TEF2*) between *Sac*I and *Xba*I sites and *CYC1* terminator (*CYC1*t) between *Xho*I and *Kpn*I sites	Mumberg et al., 1995
pRD3F	p416tef derivative that contains KanMX4 gene flanked by the *Lox*P direct repeats at *Kpn*I sites	This study
pGEMt::V1*hap4*	pGEMt easy vector in which V1*hap4* amplified with primers Hap4.u and Hap4.l from V1 strain was cloned. The gene can be excised after digestion with *Spe*I and *Bam*HI	This study
pGEMt::V1*HAP4*	pGEMt easy vector in which functional V1*HAP4* amplified with primers Hap4pro.u and Hap4ter.l from V1 strain was cloned. The gene can be excised after digestion with *Spe*I and *Bam*HI	This study
pGEMt::ck*HAP4*	pGEMt easy vector in which ck*HAP4* amplified with primers Hap4.u and Hap4.l from CenpK.113-7D strain was cloned. The gene can be excised after digestion with *Spe*I and *Bam*HI	This study
pRD3F::V1*hap4*	pRD3F plasmid in which V1*hap4* was cloned between *Spe*I and *Bam*HI sites	This study
pRD3F::ck*HAP4*	pRD3F plasmid in which the ck*HAP4* was cloned between *Spe*I and *Bam*HI sites	This study

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