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Research Article

Increasing sulphite formation in *Saccharomyces cerevisiae* by overexpression of *MET14* and *SSU1*

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Abstract

***Saccharomyces cerevisiae* produces sulphite as an intermediate product during the assimilatory reduction of sulphate to sulphide. Three genes, *MET3*, *MET14* and *MET16*, are essential for this reduction. We investigated the level of transcription of these genes in strains of *S. cerevisiae* with high, medium and low sulphite formation. The level of *MET14*- and *MET16*-mRNA varied with sulphite production, whereas the level of *MET3*-mRNA was very weak in almost all strains. We also analysed the effect of overexpression of *MET14* and *MET16* on sulphite formation. Two strains with low sulphite production were transformed with high-copy plasmids containing either or both *MET14* and *MET16*. The overexpression of these two genes leads to a two- to three-fold sulphite formation. In addition, inactivation of *MET10*, encoding a subunit of the sulphite reductase, also leads to a distinct increase in sulphite formation; however, the cells became methionine auxotroph. The overexpression of *SSU1*, a gene encoding a putative sulphite pump, yields a slight increase in sulphite accumulation, whereas overexpression of *SSU1*, together with *MET14*, increases sulphite formation up to 10-fold. Furthermore, sulphite formation strongly depends on growth conditions, e.g. yeast transformants growing in wort produce much higher amounts of sulphite when compared to growth in minimal media. The addition of glucose can also increase the sulphite formation in strains overexpressing *MET14* and/or *SSU1* under oxygen-limiting conditions, while the addition of glucose has no significant effect under aerobic conditions.**

Keywords: ***Saccharomyces cerevisiae*; sulphite formation; *MET14*; *MET16*; *SSU1***

Introduction

Sulphite is an important component in many foods and beverages due to its antioxidant and antimicrobial properties. For instance, sulphite stabilizes the flavour in beer by forming adducts with carbonyls responsible for the so-called 'cardboard flavour'. These sulphite–carbonyl complexes have a much higher flavour detection threshold than free carbonyls. *Saccharomyses cerevisiae* produces sulphite as an intermediate product during the assimilatory reduction of sulphate to sulphide, which is important for the biosynthesis of the sulphur-containing amino acids methionine and cysteine (Figure 1). Depending on the strain used and the brewing conditions, there are significant differences in the amount of sulphite produced. Because the sulphate anion is very stable, two activation steps are needed for sulphate assimilation (see Figure 1). After inorganic sulphate is taken up by the cells, it is activated into adenosylphosphosulphate (APS) by ATP sulphurylase. In the next step sulphate is again phosphorylated by APS kinase, yielding phosphoadenosylphosphosulphate (PAPS). The activated sulphate is reduced by the PAPS reductase, yielding sulphite and phosphoadenosylphosphate (PAP). Thus, sulphite formation depends on three key enzymes: ATP sulphurylase (encoded by *MET3*); APS kinase (encoded by *MET14*); and PAPS reductase (encoded by *MET16*). In the next step sulphite is reduced to sulphide by sulphite reductase, which is encoded by *MET10* and *MET5* (for review, see Thomas and Surdin-Kerjan, 1997).

It is well known that the sulphur amino acid pathway is regulated transcriptionally. The *MET* genes are repressed when methionine,

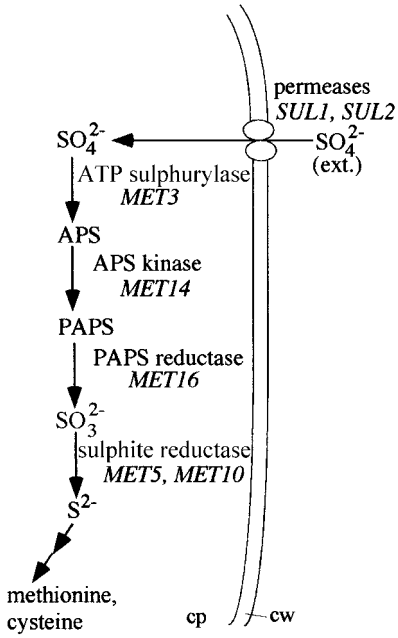

Figure 1. The assimilatory reduction of sulphate to sulphide in *S. cerevisiae* during the biosynthesis of methionine and cysteine. The structural genes and enzymes for the various steps are shown. cw, cytoplasma membrane; cp, cytoplasma; APS, adenosylphosphosulphate; PAPS, phosphoadenosylphosphosulphate

S-adenosylmethionine (SAM), homocysteine or higher concentrations of cysteine are added to the growth medium (Cherest *et al.*, 1969, 1973a, 1973b, 1985; Mountain *et al.*, 1991; Hansen and Johannesen, 2000). The structural genes are regulated by six different regulatory factors: the transcriptional inhibitor Met30p and the five positively acting factors Cbf1p, Met28p, Met31p, Met32p and Met4p, the main transcriptional activator (for review, see Thomas and Surdin-Kerjan, 1997).

The general control of amino acid biosynthesis including its transcriptional activator Gcn4p only plays a minor role. The promoters of most *MET* genes do not contain the binding site of Gcn4p. Only some genes, e.g. *MET16*, are under the control of Gcn4p, and their expression is increased when an amino acid is starved (Mountain *et al.*, 1991; O'Connell *et al.*, 1995).

Sulphate is transported into the cells by highly specific membrane transport systems. Three genes are involved in sulphate uptake: *SUL1* and *SUL2* encoding high-affinity sulphate transport proteins, and *SUL3* encoding a factor involved in the transcriptional regulation of *SUL2* (Cherest *et al.*, 1997). Sulphite enters the cells either as sulphur dioxide via an active carrier-mediated process, by simple diffusion (Stratford and Rose, 1986), or by active transport (Macris and Markakis, 1974).

Recently, Park and Bakalinsky (2000) have found a putative sulphite pump encoded by *SSU1*. It is localized in the plasma membrane. The protein encoded by *FZF1* is a transcriptional activator and interacts directly with *SSU1*. Cells lacking the Ssu1p are sensitive to sulphite, while cells overexpressing this protein are resistant. Park and Bakalinsky (2000) supposed that Ssu1p is responsible for sulphite resistance by transporting sulphite out of the cell.

Sulphite formation as part of the amino acid biosynthesis is very complex and highly regulated. In this study we are looking for the bottleneck of sulphite formation and accumulation in *S. cerevisiae* to increase sulphite production. In principle, two different ways exist to increase sulphite formation: the first is to enhance the formation of sulphite and the second is to prevent further reduction of sulphite to sulphide. We wanted to investigate both ways: the effect of an overexpression of one or two genes in combination with a decrease of the reduction of sulphite formed.

An important aspect for increasing sulphite production is the timing. If sulphite is present at an early stage of fermentation, it forms complexes with wort carbonyls and prevents the reduction of these carbonyls to the corresponding alcohols, thus influencing beer flavour negatively (Dufour, 1991). Only if sulphite formation starts at a later stage of fermentation is the desired effect achieved. Therefore, it is necessary to delay the increase of sulphite formation. We were able to show that the promoters of the genes *HSP26* and *HSP30* are suitable for an overexpression of genes at the end of the exponential and during stationary phase, respectively, in media such as wort (Donalies and Stahl, 2001). Thus, these promoters were used for a delayed increase in sulphite production.

Materials and methods

Yeast strains and growth conditions

Saccharomyces cerevisiae strains used are listed in Table 1. Yeast strains were grown in B medium (Cherest and Surdin-Kerjan, 1992), yeast nitrogen base (YNB, Difco), yeast nitrogen base with

Table 1. *Saccharomyces cerevisiae* strains used in this work

Name	IfGB-No.*	Strain	Genotype
L1	07213	S 3 B	**a**, leu1, ade2
L2	07223	S 37	α, leu, met5
L3	07236	S 2021	α, ura3, trp5, leu1, ade6, his, gal2
L6	07245	SHY 3	**a**, ura3-52, trp1, leu2, his3, ade1
L9	07239	YNN 282	α, trp, his3, ura3, lys2, ade2, gal
L10	07238	YNN 282	**a**, trp1, his3, ura3, lys2, ade2, gal
L11	07241	CG 379	α, ade5, his7, leu2, trp1, ura3

*IfGB; strain collection of the Institut für Gärungsgewerbe Berlin.

the addition of 0.1% casamino acids [YNB(C), both Difco] and wort at 28°C. The cultures were either grown under aerobic conditions in shaking Erlenmeyer flasks or under oxygen-limiting conditions in Erlenmeyer flasks (stoppered with air locks with a bubbling CO_2 outlet to exclude oxygen, with gentle stirring). For fermentation experiments, 10^7 cells/ml were inoculated from an overnight aerobic pre-culture.

Sulphite, glucose and maltose analyses

The concentration of sulphite, glucose and maltose in the fermentation supernatant was analysed by enzymatic UV methods with test-combinations for sulphite and maltose/sucrose/D-glucose, respectively (r-Biopharm) according to the manufacturer's instructions. The sulphite test measures the total amount of sulphite (free and carbonyl-bound sulphite).

mRNA analyses

Total RNA was extracted from yeast cells grown in wort. 50–100 ml culture were washed, resuspended in 700 µl extraction buffer (0.5 M NaCl, 0.2 M Tris, 10 mM EDTA, pH 7.5) and 20 µl β-mercaptoethanol were added. The cells were frozen in pre-cooled Teflon cylinders using liquid nitrogen, 750 µl phenol : chloroform : isoamylalcohol (25 : 24 : 1) was added and the cells were broken up with two steel balls in a Micro-Dismembrator II (Braun, Melsungen, Gemany) for 2 min (amplitude: 1.2–1.5 cm). The RNA was separated from the cell debris by centrifugation for 1 h at $18\,000 \times g$ and further purified by phenol/chloroform extraction. The RNA was precipitated with 1/10 volume 1 M acetate and 0.7 volume ethanol (96%), washed with 70% ethanol, and dissolved in 100–200 µl autoclaved DEPC–H_2O (0.1% diethylpyrocarbonat). 30 µg total RNA was separated for each probe in 1.5% agarose gels and transferred to nylon membranes (Hybond™-N, Amersham). The ethidium bromide-stained 18S and 28S rRNA was used as the loading control. The DNA probes were prepared from the genomic DNA of *S. cerevisiae* strain L6 by PCR, using specific primers for coding sequences of investigated genes and labelled with [α-^{32}P]dATP. Aliquots of unlabelled DNA probes were dotted on membranes as the positive control. Blots were hybridized with DNA probes at 60°C. After stripping (0.1% SDS, 100°C, 5 min), the membranes were hybridized with *ACT1* probes as a standard. Hybridized membranes were exposed to an X-ray film for 1–7 days.

Construction of plasmids and transformation of *S. cerevisiae*

YEP26-14 was constructed by fusing the *Nco*I–*Hin*dIII PCR fragment of the coding sequence of *MET14* (primers: 5′-CAC CCA TGG CTA CTA ATA TTA CTT G-3′, 5′-TAT AAG CTT GCA AGC TCG GTT GAA CAC-3′) to the 1 kb *Eco*RI–*Nco*I fragment of YEP26lacZ (Donalies and Stahl, 2001) containing the *HSP26* promoter. This fusion gene was inserted as an *Eco*RI–*Hin*dIII fragment in the YEP358-2 replicative vector carrying the *URA3* gene as selection marker. YEP358-2 was constructed from YEP358 (Myers *et al*., 1986) by removing the *lacZ* gene by *Pvu*II restriction. Genomic DNA of *S. cerevisiae* strain L6 was used as a template for all PCRs. The YEP26-16 was constructed by fusing the 1 kb *Bam*HI–*Nco*I fragment of YEP26lacZ containing the *HSP26* promoter to the *Nco*I-*Hin*dIII PCR fragment of the coding sequence of *MET16* (primers: 5′-AAT CCA TGG GGA AGA CCT ATC ATT TGA ATA ATG-3′, 5′-GGT GGA AGC TTG GTT TTT ATA G-3′) and inserting as a *Bam*HI–*Hin*dIII fragment in the YEP358-2 replicative vector. YEP30-14 was constructed in the same way as YEP26-14 using the 1.1 kb *Eco* RI–*Nco*I fragment of YEP30lacZ (Donalies and Stahl, 2001) containing the *HSP30* promoter instead of the *HSP26* fragment. The fusion genes were also ligated in the pBluescriptSK$^+$ phagemid vector (pBSK$^+$, Stratagene) yielding pBSK$^+$/HSP26–MET14, pBSK$^+$/HSP26–MET16 and pBSK$^+$/HSP30–MET14. The *Kpn*I–*Spe*I fragment of pBSK$^+$/HSP30–MET14, which contains the coding sequence of *MET14* under

control of the *HSP30* promoter, was ligated into YEP26-16 to yield YEP30-14/26-16. For YEPS1, the *Eco*RI–*Bam*HI fragment of pHP18 (Park *et al*., 1999) containing the *SSU1* ORF and promoter sequence was inserted into YEP358-2. The *Xho*I–*Bam*HI fragment of pBSK$^+$/HSP26–MET14 was ligated into the *Bam*HI–*Sal*I digested YEPS1 to yield YEPS1/26-14. All plasmids constructed are listed in Table 2.

Escherichia coli XL1-blue was used to select the plasmid constructs. *S. cerevisiae* strains were transformed by electroporation as described by Becker and Guarente (1991). The transformants were selected on YNB without uracil.

Disruption of *MET10*

The *MET10* gene was inactivated using the cre/*lox*P disruption cassette as described in Güldener *et al*. (1996) (primers: 5′-AAC GAG ATG CCA GTT GAG TTT GCT ACC AAT CCT TTT GGC GCA GCT GAA GCT TCG TAC GC-3′, 5′-GCC AGA ATG TCT TGC AAA GCT TGA GTA ATA TCT GGA ACT GGC ATA GGC CAC TAG TGG ATC TG-3′). *S. cerevisiae* strains were transformed with 5 µg of the disruption cassette by electroporation as described by Becker and Guarente (1991). The transformants were selected on YE (0.5% yeast extract, 2% glucose) supplemented with 75 µg/ml G418.

Results

Sulphite formation and transcriptional analysis of *MET3*, *MET14* and *MET16* in different *S. cerevisiae* strains

In a first step we screened several strains of *S. cerevisiae*, which are able to use maltose, the main carbon source in wort. Twelve strains, which could grow on YNB plates supplemented with maltose as sole carbon source, were selected. These strains were grown in wort and after 24 h the sulphite content in the supernatant was measured. Seven strains, which usually produce different amounts of sulphite, were chosen (L1, L2, L3, L6, L9, L10 and L11; see Table 1), and the transcriptional levels of *MET3*, *MET14* and *MET16* were investigated.

During fermentation in wort the highest accumulation of sulphite after 30 h was observed for strains L1(8 mg/l), L2 and L6 (10–12 mg/l), while L3, L9, L10 and L11 only formed very small amounts of sulphite (1–3 mg/l) (Figure 2A). Northern analyses showed that the mRNA level of *MET14* and *MET16* was strongly increased in the strains showing the highest rate of sulphite production

Table 2. Plasmids for overexpression of *MET14*, *MET16* and *SSU1* in *S. cerevisiae*

Plasmid	ORF	Promoter
YEP26-14	*MET14*	*HSP26*
YEP26-16	*MET16*	*HSP26*
YEP30-14	*MET14*	*HSP30*
YEP30-14/26-16	*MET14*	*HSP30*
	MET16	*HSP26*
YEPS1	*SSU1*	*SSU1*
YEPS1/26-14	*SSU1*	*SSU1*
	MET14	*HSP26*

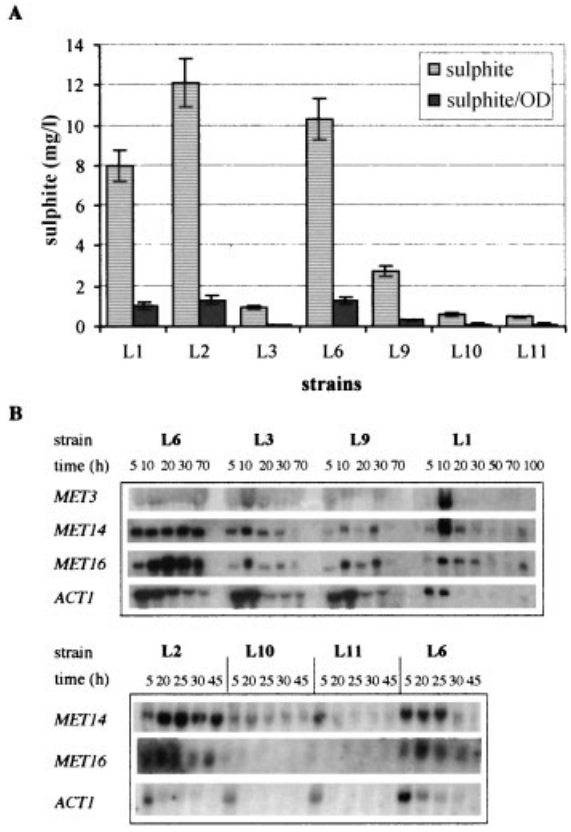

Figure 2. Sulphite formation after 30 h (A) and Northern analyses (B) of *MET3*, *MET14* and *MET16* in different *S. cerevisiae* strains in wort. Cells were grown under oxygen-limiting conditions at 28°C with slight stirring. *ACT1* probes were used as a standard. Hybridized membranes were exposed to an X-ray film for 1 day (*ACT1*), 2 days (*MET14* and *MET16*) or 5 days (*MET3*)

(Figure 2B). Interestingly the transcription level of *MET3* was very low in all strains analysed, except for L1. Only in this strain was the transcription of *MET3* very strong after 10 h. These data suggest that an overexpression of *MET14* and *MET16* might be useful to increase sulphite formation even in low-sulphite producing strains.

Overexpression of *MET14* and *MET16*

Four high-copy plasmids were constructed by ligating the coding sequences of *MET14* and *MET16* downstream of the *HSP26* and the *HSP30* promoter, respectively. All plasmids are listed in Table 2. The low-sulphite producing strains L3 and L10 were transformed with each of the plasmids. The transformants carrying the YEP26-14 and YEP30-14 plasmids (overexpression of *MET14*) show an increase in sulphite formation up to 14 mg/l (seven-fold when compared to the untransformed strain) during aerobic grow in wort (Figure 3A). Interestingly, in the yeast transformed with YEP26-16 (overexpression of *MET16*), no increase in sulphite production could be observed. The strain L3 transformed with YEP30-14/26-16 (*MET14* and *MET16*) shows also a seven-fold increase in sulphite formation in wort, indicating that additional overexpression of *MET16* yields no significant further increase in sulphite accumulation. The transformants of the strain L10 yielded similar results (data not shown).

Since wort is a complex medium with a variable composition from batch to batch, we repeated the experiments in well-defined minimal media (B medium and YNB, respectively). However, the growth rate is strongly reduced in these media. The maximum optical density measured at 600 nm (OD_{600nm}) was between 3 and 5, compared with OD_{600nm} = 14–18 in wort. Since sulphite formation is strongly dependent on metabolic activity, the strains produce lower amounts of sulphite in minimal media. Nevertheless we observed an increase in sulphite formation by overexpression of *MET14* also in minimal media (data not shown). 0.1% casamino acids, which contain non-repressing amounts of amino acids, were added to improve cell growth. In YNB with 0.1% casamino acids [YNB(C)] cell growth is improved (OD_{600nm} = 7–8) and the formation of sulphite by L3/YEP26-14 and L3/YEP30-14/26-16 is increased two- to three-fold (2–3 mg/l) when compared to the wild-type (wt) strain (Figure 3A). In order to compare sulphite formation in YNB(C) and wort, the amount of sulphite in the supernatant was normalized to OD_{600nm} of the culture. Figure 3B shows that in cells overexpressing *MET14* the sulphite per OD_{600nm} is much higher in wort (two- to three-fold) than in the minimal medium YNB(C).

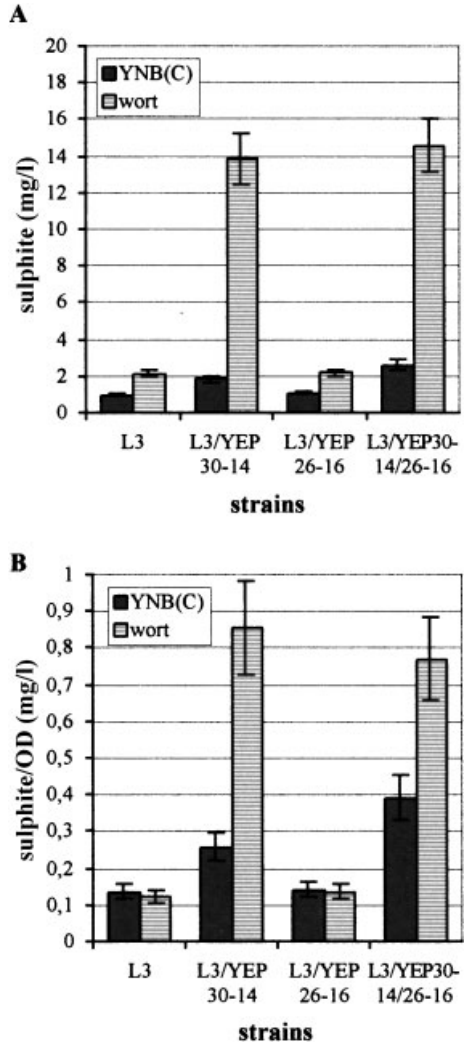

Figure 3. Sulphite formation of the *S. cerevisiae* strain L3 overexpressing either *MET14*, *MET16* or both. (A) Sulphite in the supernatant and (B) sulphite in the supernatant normalized to the optical density at 600 nm (OD). Cells were grown aerobically in YNB(C) and wort in shaking flasks at 28°C for 24 h

Overexpression of *SSU1* and *MET14*

Although sulphite formation can be increased by overexpression of *MET14*, accumulation of sulphite

is limited, because it is immediately reduced by an active sulphite reductase. This enzyme consists of two subunits encoded by the genes *MET5* and *MET10*. Therefore, we inactivated *MET10* in two wt strains using the cre/*lox*P disruption cassette (Güldener *et al.*, 1996). The *met10* strains show an increase in sulphite formation up to 10-fold (data not shown). However, the strains became methionine auxotroph, the growth is reduced and the sulphite accumulation starts at the beginning of the fermentation.

Park and Bakalinsky (2000) have published a putative sulphite pump encoded by *SSU1*. Thus, we wanted to test the effect of *SSU1* overexpression on sulphite formation. We ligated the single *SSU1* gene (YEPS1), and both *SSU1* and *MET14*, under the control of the HSP26 promoter (YEPS1/26-14). The strains L3 and L10 were transformed with these plasmids and sulphite formation of different clones in B medium (data not shown), YNB(C) and wort were measured (Figure 4A). During aerobic grow of the L3 transformants in minimal media the overexpression of *SSU1* alone leads to a two-fold increase in sulphite accumulation (B medium, 1 mg/l; YNB(C), 3 mg/l), while overexpression of *SSU1* and *MET14* increases sulphite formation up to 10-fold (B medium, 6 mg/l; YNB(C), 8 mg/l). In wort the L3/YEPS1 shows the same sulphite formation as the wt, whereas L3/YEPS1/26-14 forms 17–19 mg/l sulphite (seven- to nine-fold). Figure 4B shows sulphite in the supernatant normalized to the OD_{600nm} of the culture. It is clearly seen in this figure that the overexpression of *MET14* leads to a higher increase in sulphite formation in wort than in YNB(C), whereas the overexpression of *SSU1* only has a slight effect in wort. The L10 transformants yielded similar results (data not shown).

To show the influence of methionine on gene expression, we investigated sulphite formation in strains transformed with YEPS1, YEP26-14 and YEPS1/26-14 in B medium adding no, 0.5 mM and 1 mM methionine, respectively. Sulphite accumulation in L3/YEP26-14, L3/YEPS1 and L3/YEPS1/26-14 decreased by 40% in medium containing 0.5 or 1 mM methionine.

Fermentation (oxygen-limiting conditions) with different L3-transformants in B medium yield a two-fold sulphite formation when either *MET14* or *SSU1* is overexpressed (Figure 5). In contrast, the combination of *SSU1* and *MET14* expression significantly increase sulphite production up to 10-fold (4 mg/l) during the stationary phase.

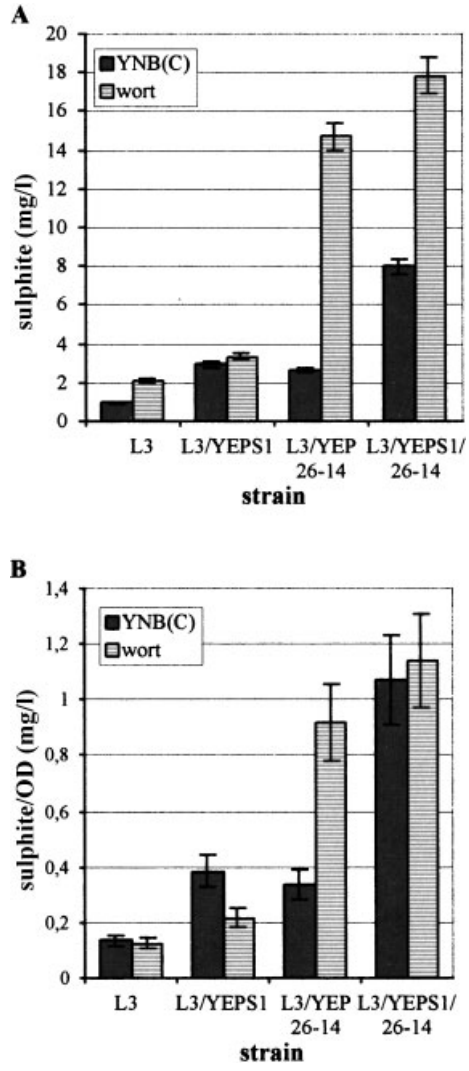

Figure 4. Sulphite formation of the *S. cerevisiae* strain L3 overexpressing either *SSU1*, *MET14* or both. (A) Sulphite in the supernatant and (B) sulphite in the supernatant normalized to the OD_{600nm} Cells were grown aerobically in YNB(C) and wort in shaking flasks at 28°C for 24 h

During fermentation in wort the strains reach the stationary phase at lower cell densities (OD_{600nm} = 8–10) when compared to aerobic growth in shaking flasks (OD_{600nm} = 14–18) and does not form significant amounts of sulphite (data not shown). The analysis of sugar utilization showed that the strains only metabolized traces of wort maltose, although the strains were able to grow with maltose as the sole carbon source on minimal medium YNB. Therefore, glucose (5% and 10%) was added to the

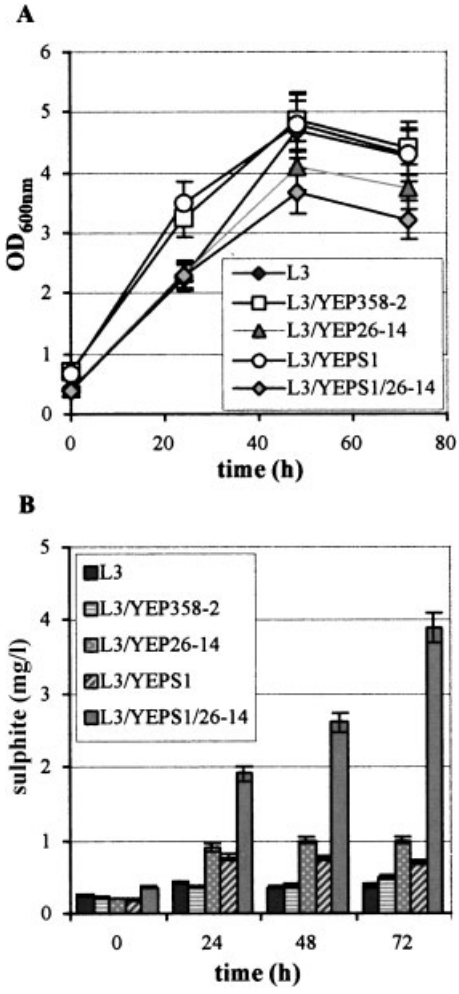

Figure 5. Growth kinetics (A) and sulphite formation (B) of *S. cerevisiae* strain L3 and different transformants of that strain during fermentation in B medium. Cells were grown under oxygen-limiting conditions at 28°C with slight stirring

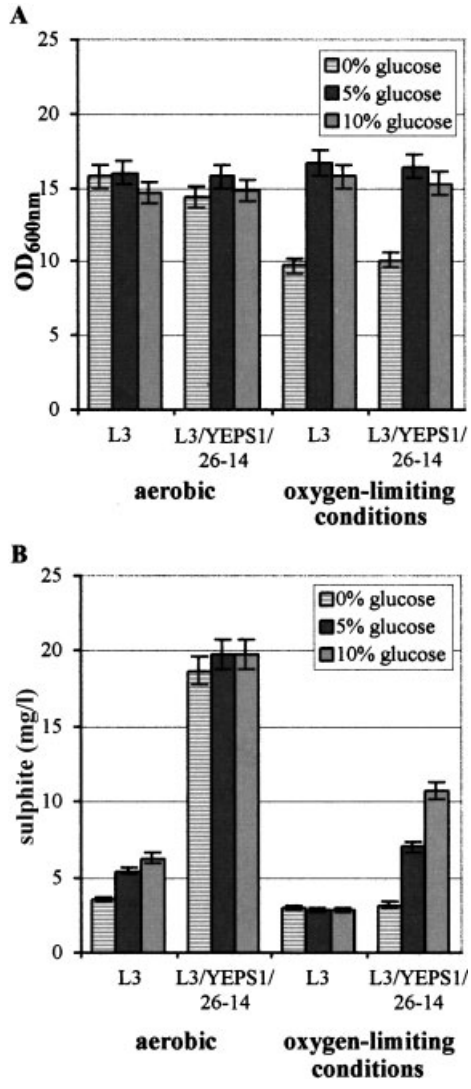

Figure 6. Growth (A) and sulphite formation (B) of *S. cerevisiae* strain L3 and L3/YEPS1/26-14 in wort under aerobic and oxygen-limiting conditions dependent on glucose addition (0%, 5%, 10%). Cells were grown at 28°C for 24 h either aerobically in shaking flasks or under oxygen-limiting conditions with slight stirring

wort and cell density and sulphite formation depending on glucose content were measured (Figure 6). Cell densities are higher in wort with additional glucose under oxygen-limiting conditions. Sulphite formation is not influenced in the wt strain, while the strain L3/YEPS1/26-14 shows a significant increase in sulphite formation depending on glucose content. Glucose content has no significant influence on growth under aerobic conditions, although sulphite formation is slightly increased by adding glucose in the untransformed strain.

Discussion

In *Saccharomyces cerevisiae* three key enzymes are responsible for sulphite formation: ATP sulphurylase, APS kinase, and PAPS reductase, encoded by *MET3*, *MET14* and *MET16*, respectively. The level of *MET14*- and *MET16*-mRNA correlates with sulphite formation, while the transcription of *MET3* is very low in almost all strains analysed. It

seems that the low *MET3*-mRNA level is sufficient to provide a high sulphite production. We therefore concentrated on the analysis of *MET14* and *MET16* overproduction on sulphite formation. The two genes were expressed under the control of the *HSP26* or *HSP30* promoter, providing a strong transcription only at the end of the exponential phase and during the stationary phase (Donalies and Stahl, 2001).

Overexpression of *MET14* increases sulphite formation in two low sulphite-producing strains, while overexpression of *MET16* alone has no effect. Combined overexpression of *MET14* and *MET16* did not lead to a significant further increase, indicating that a high level of *MET14* mRNA is sufficient for effective sulphite accumulation. Korch *et al.* (1991) found that overexpression of *MET14* (under control of its own promoter) increases sulphite formation significantly only in a *met5* mutant (inactive sulphite reductase), but not in a *MET5* prototrophic laboratory strain. Furthermore, Johanneson (1994, personal communication) showed that a *MET14* gene under the control of the *TPI* promoter leads to a stronger transcription. In a *met10* strain (inactive sulphite reductase) this promoter exchange caused a slight increase in sulphite production, whereas in a strain with active sulphite reductase no influence could be observed. These findings confirm that a high expression of *MET14* causes an increase in sulphite formation. However, this effect is strongly dependent on the promoter, which controls *MET14*, and on the copy-number and the strain used. Our results show that the overexpression of *MET14* under the control of a strong promoter, e.g. *HSP26* or *HSP30*, leads to a significant increase in sulphite formation in two different laboratory strains, even with an active sulphite reductase.

Sulphite accumulation is limited by an active sulphite reductase converting sulphite to sulphide. It is probable that the overexpression of *MET14* increases not only the concentration of sulphite, but also that of sulphide. Sulphide is responsible for H_2S formation, an undesired by-product during the brewing process. A way to prevent the reduction of sulphite to sulphide is to inactivate the sulphite reductase by disruption of either the *MET5* or the *MET10* gene. The *MET10* gene knockout yields a 10-fold increase in sulphite accumulation in two independent wt strains; however, the strains became methionine auxotroph. Hansen and Kielland-Brandt (1996) inactivated two divergent *MET10* genes in a brewer's yeast. This mutation leads to an early and very large accumulation of sulphite. However, the *met10* strain shows a slower metabolization of carbohydrates from the wort and a lower cell density in comparison to the prototrophic strain. Moreover, Dufour (1991) showed that sulphite formation in the cells already starts at the beginning of fermentation preventing the reduction of wort carbonyls to the corresponding alcohols during the brewing process. Furthermore, the dimethyl sulphoxide, α-acetolactate and diacetyl formation is increased using the *met10* yeast (Hansen and Kielland-Brandt, 1996). These components cause off-flavour in beer. Thus, to avoid those problems we looked for an alternative way to prevent the prompt reduction of the sulphite formed.

Since Park and Bakalinsky (2000) have published a putative sulphite pump, encoded by *SSU1*, a new model of sulphite metabolism could be developed (Figure 7). We used this putative sulphite pump to increase sulphite efflux and so prevent further reduction of sulphite in the cell. Overexpression of

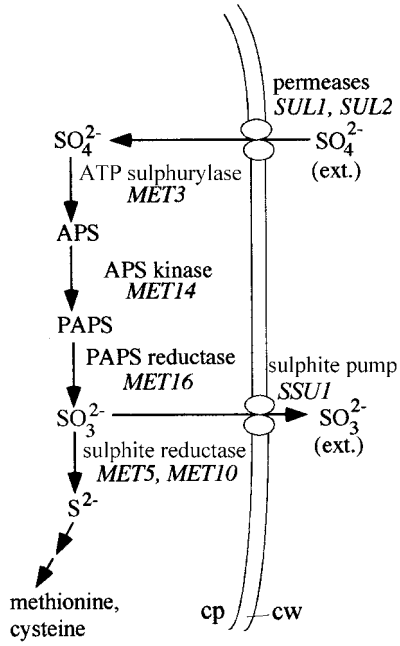

Figure 7. Proposed model for the sulphite formation and transportation in *S. cerevisiae* during the biosynthesis of methionine and cysteine. Sulphate uptake is mediated by permeases and the formed sulphite is secreted by a sulphite pump. cw, cytoplasma membrane; cp, cytoplasma; APS, adenosylphosphosulphate; PAPS, phosphoadenosylphosphosulphate

SSU1 leads to a double sulphite formation in minimal media but has only a weak effect in wort. These data suggest that the *SSU1* gene might be repressed by a component in the complex wort medium. A change of the *SSU1* promoter might help to avoid this repression. In contrast, overexpression of *SSU1* and *MET14* together leads to a significant increase in sulphite formation (up to 10-fold).

The amount of sulphite strongly depends on the medium and growth conditions. The *MET* genes, including the genes coding for sulphate permeases, are repressed when methionine or higher concentrations of cysteine are found in the growth medium (Cherest *et al*., 1969, 1973a, 1973b, 1985; Mountain *et al*., 1991; Hansen and Johannesen, 2000). In minimal media with non-repressive amounts or without methionine and cysteine the genes for sulphite formation are derepressed, but the cells are unable to grow to high densities. Since sulphite formation is strongly coupled with the cell growth the amounts of sulphite formed are reduced. For the same reason, the sulphite formation under aerobic conditions is much higher than under oxygen-limiting conditions. During fermentation in wort, the addition of glucose results in better growth and increased sulphite formation in the transformed strains significantly.

To summarize, sulphite formation in transformed strains is strongly dependent on growth conditions, and the yeast strains used. Although the absolute amount of sulphite differs, the proportion between the different transformants is always the same: highest sulphite accumulation is achieved in strains overexpressing *MET14* and *SSU1*, followed by overexpression of *MET14* alone. Thus, an increase of APS kinase activity and an enhanced transport of the formed sulphite out of the cell should be the most suitable way to increase sulphite formation during fermentation. The amount of sulphite produced and the point in time that sulphite is formed is controlled by the promoter fused to *MET14* and the copy-number. Our strategy has the advantage that no gene inactivation is needed, which would result in strong interference in the metabolism accompanied with undesired side-effects.

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References

Becker DM, Guarente L. 1991. High-efficiency transformation of yeast by electroporation. *Methods Enzymol* **194**: 182–187.

Cherest H, Davidian J-C, Thomas D, Benes V, Ansorge W, Surdin-Kerjan Y. 1997. Molecular characterization of two high affinity sulphate transporters in *Saccharomyces cerevisiae*. *Genetics* **145**: 627–635.

Cherest H, Eichler F, de Robichon-Szulmajster H. 1969. Genetic and regulatory aspects of methionine biosynthesis in *Saccharomyces cerevisiae*. *J Bacteriol* **97**: 382–336.

Cherest H, Surdin-Kerjan Y. 1992. Genetic analysis of a new mutation conferring cysteine auxotrophy in *Saccharomyces cerevisiae*: updating of the sulphur metabolism pathway. *Genetics* **130**: 51–58.

Cherest H, Surdin-Kerjan Y, Antoniewski J, de Robichon-Szulmajster H. 1973a. S-adenosyl methionine-mediated repression of methionine biosynthetic enzymes in *Saccharomyces cerevisiae*. *J Bacteriol* **114**: 928–933.

Cherest H, Surdin-Kerjan Y, Antoniewski J, de Robichon-Szulmajster H. 1973b. Effects of regulatory mutations upon methionine biosynthesis in *Saccharomyces cerevisiae*: Loci *eth2-eth3-eth10*. *J Bacteriol* **115**: 1084–1093.

Cherest H, Thao NN, Surdin-Kerjan Y. 1985. Transcriptional regulation of the *MET3* gene of *Saccharomyces cerevisiae*. *Gene* **34**: 269–281.

Donalies UEB, Stahl U. 2001. Phase-specific gene expression in *Saccharomyces cerevisiae*, using maltose as carbon source under oxygen-limiting conditions. *Curr Genet* **39**: 150–155.

Dufour J-P. 1991. Influence of industrial brewing and fermentation working conditions on beer SO_2 level and flavour stability. In *European Brewery Convention, Proceedings of the 23rd Congress, Lisbon, 1991*. IRL Press at Oxford University Press: Oxford, UK; 209–216.

Güldener U, Heck S, Fiedler T, Beinhauer J, Hegemann J. 1996. A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res* **24**: 2519–2524.

Hansen J, Johannesen PF. 2000. Cysteine is essential for transcriptional regulation of the sulphur assimilation genes in *Saccharomyces cerevisiae*. *Mol Gen Genet* **263**: 535–542.

Hansen J, Kielland-Brandt MC. 1996. Inactivation of *MET10* in brewer's yeast specifically increases SO_2 formation during beer production. *Nature Biotechnol* **14**: 1587–1591.

Johannesen PF. 1994. Increasing the flux in the sulfur assimilatory pathway. MSc thesis, University of Copenhagen, Copenhagen (unpublished).

Korch C, Mountain HA, Gyllang H, Winge H, Brehmer P. 1991. A mechanism for sulphite production in beer and how to increase sulphite levels by recombinant genetics. In *European Brewery Convention, Proceedings of the 23rd Congress, Lisbon,*

1991. IRL Press at Oxford University Press: Oxford, UK; 201–208.

Macris BJ, Markakis P. 1974. Transport and toxicity of sulphur dioxide in *Saccharomyces cerevisiae* var *ellipsoideus*. *J Sci Food Agric* **25**: 21–29.

Mountain HA, Byström AS, Larsen J, Korch C. 1991. Four major transcriptional responses in the methionine/threonine biosynthetic pathway of *Saccharomyces cerevisiae*. *Yeast* **7**: 781–803.

Myers AM, Tzagoloff A, Kinney DM, Lusty CJ. 1986. Yeast shuttle and integrative vectors with multiple cloning sites suitable for construction of *lacZ* fusions. *Gene* **45**: 299–310.

O'Connell KF, Surdin-Keran Y, Baker RE. 1995. Role of *Saccharomyces cerevisiae* general regulatory factor CP1 in methionine biosynthetic gene transcription. *Mol Cell Biol* **15**: 1879–1888.

Park H, Bakalinsky AT. 2000. *SSUI* mediates sulphite efflux in *Saccharomyces cerevisiae*. *Yeast* **16**: 881–888.

Park H, Lopez NI, Bakalinsky AT. 1999. Use of sulphite resistance in *Saccharomyces cerevisiae* as a dominant selectable marker. *Curr Genet* **36**: 339–344.

Thomas D, Surdin-Kerjan Y. 1997. Metabolism of sulphur amino acids in *Saccharomyces cerevisiae*. *Microbiol Mol Biol Rev* **61**: 503–532.

Stratford M, Rose AH. 1986. Transport of sulphur dioxide by *Saccharomyces cerevisiae*. *J Gen Microbiol* **132**: 1–6.