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Improvement and analysis of selenate resistant *Schizosaccharomyces pombe* mutants defective in sulphur metabolism

PhD thesis

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Budapest
2004

PhD School/Program

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1 INTRODUCTION

1.1 Introduction

It has long been known that certain strains of the fission yeast *Schizosaccharomyces pombe* are able to metabolize high amount of L-malic acid via the malo-ethanolic fermentation pathway. Malo-ethanolic fermentation (MEF) by *Schiz. pombe* could serve as an alternative method for biological deacidification of grape must or wine instead of the malo-lactic fermentation (MLF) carried out with lactic acid bacteria, which process has several difficulties. Numerous studies were performed on *Schiz. pombe* from technological aspects, but its applicability to vinification has been found controversial. The main disadvantage of the fermentation process is that many of the tested strains were found to produce off-flavours, including H_2S and other sulphur containing compounds (mercaptans and disulphides).

This study is a part of a breeding program aiming to improve a suitable strain for enological porposes, to achieve a yeast strain with high malic acid fermentation rate, reduced or eliminated sulfid (H_2S) formation capability and excellent flocculation properties. In our research team Geleta (1996) has found different groups of *Schiz. pombe* and *Schiz. octosporus* strains with highly variable phenotypic characters on the basis of physiological features as ethanol tolerance, malic acid degradation rate and H_2S production of the isolates, furthermore developed a flocculent strain which tolerated 12% of ethanol content in wine and metabolized considerable amount of malic acid but H_2S formation of this strain has not been controled.

The amount of off-flavours as H_2S and its sulphur-containing derivates produced by the strains during fermentation is influenced by several factors (such as nitrogen supply and others) but mostly by the genetic characteristic of the yeast strain applied.

Although much is known about physiology and genetics of *Schiz. pombe*, its sulphur metabolism has been poorly studied. Sulphate metabolism has been well studied in yeast *Saccharomyces cerevisiae* and in certain filamentous fungi, such as *Neurospora crassa* and *Aspergillus nidulans.*

Sulphate, the major sulphur source in many organism, transported into cells by specific membrane transport systems. After accumulation sulphate is enzymatically reduced to sulphide by the sulphate assimilation pathway and than incorporated into organic compounds. In yeast the sulphate assimilation pathway begins with the activation of sulphate

anions in two seqential reactions: the first transfers the adenosyl-phosphoryl moiety of ATP to sulphate, yielding adenylyl sulphate (APS = 5'-adenosin-phospho-sulphate), which is in turn phosphorylated to yield phosphoadenylyl sulphate (PAPS = 3'-phospho-adenosin-5'-phospho-sulphate). The enzymes catalysing these two reactions are ATP sulphurylase and APS kinase, respectively. For cysteine and methionine biosynthesis, activated sulphate is sequentially reduced by PAPS reductase to sulphite which is in turn further reduced to sulphide by sulphite reductase. At the end of this process, the reduced sulphur atom can be incorporated into carbon chains.

Schiz. pombe, in contrast to *S. cerevisiae* and *Asp. nidulans*, lacks cystathionine *β*-synthase and cystathionine *γ*-lyase, two enzymes of the reverse transsulphuration pathway from methionine to cysteine (Brzywczy and Paszewski, 2002). This suggest that *Schiz. pombe* is not able to metabolize methionine efficiently to cysteine and as a consequence, methionine can not serve as an efficient sulphur source for this fungus.

Selenate is a toxic analogue of sulphate, its transport into the cell and its metabolism connected to the sulphate pathway. Arst (1968) found that certain sulphate-non-utilizing mutants of *Asp. nidulans* showed strong resistance to selenate simultaneously. These mutants belonged to two genetic complementation groups: *sB* and *sC*. *sB* gene was coding for sulphate permease and sC gene for ATP sulphurylase enzymes. *sB*$^-$ mutants have also gained chromate resistant phenotype, while *sC*$^-$ mutants retained the same degree of sensitivity as the wild type. PAPS reductase deficient mutants had only a weak selenate tolerance, while deficiency in APS kinase caused hypersensitivity to selenate. Selenate- and chromate resistant mutants of *S. cerevisiae* were also isolated and studied by Breton and Surdin-Kerjan (1977). Mutation in any of the first three genes of the sulphate reduction pathway resulted in resistance to selenate, but ATP sulphurylase deficient mutants tolerated 20-50 times more selenate than the mutants lacking APS kinase or PAPS reductase activity.

1.2 Objectives

- characterising yeast strain isolates belonging to the *Schizosaccharomyces* genus by molecular typing methods such as Random Amplified Polimorphic DNA (RAPD-PCR) analysis and Restriction Fragment Length Polymorphism of Amplified rDNA sequences (RFLP of rDNA / "ribotyping") to answer the question which correlation could be found between resulted genotipic fingerprints and the phenotypic characters investigated by Geleta (1996) before (growth rate, malic acid degradation rate and H_2S production)

- investigate sulphur metabolism in *Schiz. pombe* by inducing, isolating and analysing selenate resistant mutants defective in any steps of the sulphate assimilation pathway,
- selecting non-H_2S-forming *Schiz. pombe* strain with adequate malic acid fermentation rate for enological purposes

2 MATERIALS AND METHODS

2.1 Strains used

Strains used in this study were authentic *Schiz. pombe* strains (L.972 h^- = CBS 7264, L.975 h^+ = CBS 7265) and their auxotrophic mutants (0-82 h^- *ade5*, 0-44 h^- *arg4*, h^+ *leu1*, h^+ *lys1,* etc.), as well as *Schizosaccharomyces* strains from different culture collections, most of them primarilly isolated from grape juice and wine. Strains B573, B578, B579 were isolated from must preserved with sulphure-dioxide.

2.2 Culture media

Yeast strains were cultivated in the YEPD complete medium (1% glucose, 0.5% yeast extract, 0.5% peptone), or in a minimal medium (1% glucose, 0.5% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 1 ml/L Wickerham vitamin solution) supplemented with the required growth components (adequate amino acids). Composition of *sulphur-free minimal medium*: 1% glucose, 0.5% NH_4Cl, 0.1% KH_2PO_4, 0.05% $MgCl_2.H_2O$. For inducing sexual hibridization *YED agar medium* (1% glucose, 0.5% yeast extract, 2% agar) and *SPA agar medium* (0.1% KH_2PO_4, 1% glucose, 1 ml/L Wickerham vitamin solution, 2% agar) was used.

2.3 Brief review of experimental methods

DNA isolation was performed as published by Hoffman and Winston (1987).

For *RAPD-PCR analysis* 4 random primers (P1, P2, P3, P4) of different GC mol% and different optimal annealing temperature ($topt_{AN}$ = 36 - 42°C) have been used. Thermo-parameters of the PCR reaction were the following: pre-denaturation 94 °C for 5 minutes, 35 amplification cycles as 94 °C for 30 sec, $topt_{AN}$ for 45 sec, 72 °C for 45 sec and final extension at 72 °C for 7 minutes.

For RFLP of rDNA sequences ("ribotyping") method, an rDNA sequence was amplified flanked by the specific primers ITS4 and NS1 (White et al., 1990). This amplified rDNA sequence which contained the whole nuclear small rDNA, the 5.8S rDNA, two ITS regions and a small part of the nuclear large rDNA genes has been digested with 4 restriction enzymes, such as *Hae*III, *Msp*I, *Scrf*I and *Sau*3AI.

DNA fragments were separated by gel electrophoresis in agarose gel and visualized by ethidium-bromide in UV light. Results (fingerprints) of RAPD-PCR analysis and ribotyping have been analysed, dendograms created using the software "Molecular Analyst Fingerprint" of BIO-RAD.

Mutagenic treatments have been performed by *UV irradiation* of cell suspensions (10^7 cells/ml) in Petri dishes with a germicidal lamp and by treating cells in 30 mg/ml *ethyl-methan-sulfonate (EMS)* or 25 μg/ml *N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)* solutions. Chemical mutagens were inactivated by $Na_2S_2O_3$ solution. Mutants were isolated at 5-10% survival rate.

For the *isolation of selenate resistant mutants*, 0.4 mM sodium selenate was added to YEPD agar media. The minimal inhibitory concentration (MIC) of sodium selenate were obtained 0.1-0.2 mM at the different strains.

Sulphate utilization of the mutants was determined by their growth ability on a minimal medium containing 0.5% ammonium sulphate as the sole sulphur source. A minimal medium containing 0.01% cysteine as a generally utilisable sulphur source was used as positive control.

For determination of chromate-resistant property of selenate resistant mutants growth of the strains was tested at 2.5, 3.0 and 3.5 mM K_2CrO_4 concentrations on YEPD media (MIC value of K_2CrO_4 proved to be 2.5 mM).

Complementation analysis of selenate resistant mutants has been performed *by sexual hibridization* of sulphate-non-utilizing mutants with complementing auxotrophic mutations and opposite mating types.

Sulphate transport of cells was followed by the uptake of labelled ^{35}S-containing sulphate. Radioactivity of cells has been measured by a Packard scintillation counter.

Utilization patterns of sulphur sources have been determined by testing the growth of the strains in the presence of inorganic sulphur compounds as sulphate, thiosulphate, sulphite and organic compounds as glutathione, cysteine, methionine.

The *malic acid content* was determined by the enzymatic malic-acid kit of Boehringer-Mannheim.

For the *detection of hydrogen sulphide*, a semiquantitative method (blackening of paper strips saturated with lead acetate) and a precise analytical method has been adapted, where the liberated H_2S was trapped in $Cd(OH)_2$ solution and introduced into a blue color reaction (Jiranek et al., 1995). Color intensity is in direct ratio with amount of trapped hydrogen sulphide.

For fast comparison of *fermentation aroma profile,* a solid phase microextraction (SPME) sampling has been developed appliing a PDMS (polydimethylsiloxane) apolar fiber. Separation and identification of volatile aroma components has been carried out by GC-MS method.

3 SUMMARY OF RESULTS

RAPD-PCR analysis of *Schiz. pombe* and *Schiz. octosporus* strains resulted two clusters with 30% similarity, which corresponded to this two species. Within the species *Schiz. pombe* strains were separated into two big clusters – group ”A” and group ”B” – , while 2 strains separated as individuals. Three *Schiz. pombe* strains – B 573, B 578, B 579 – which were isolated from sulphited must, proved to be completely identical according to their RAPD patterns. This correlated well with their other physiological properties: high L-malate fermenting rate, short generation time and excellent ethanol tolerance. Strains belonged to the group ”B” metabolize high amount of malic acid while strains belonging to group ”A” are low in malic acid consumption.

RFLP analysis of rDNA sequences resulted three distinct clusters of strains with low degree of similarity, which corresponded to the currently accepted three species of the genus. The restriction enzymes *Scrf* I and *Msp*I resulted only the separation of *Schiz. japonicus* from the other *Schizosaccharomyces* species, while restriction enzymes *Hae* III and *Sau*3AI differentiated also the *Schiz. octosporus* and *Schiz. pombe* strains. Within each of the species ribotyping did not show any further difference, indicating that this method is excellent for identification at the species level. We could not find however any clustering of the formerly delineated varieties of *Schiz. pombe* (*var. pombe* and *var. malidevorans*).

By *mutagenic treatment* of different prototrophic and auxotrophic *Schiz. pombe* strains with UV irradiation, MNNG- and EMS-treatment, *selenate resistant mutants were induced and isolated* on YEPD agar plates containing 0.4 mM sodium selenate. All of them tolerated 50 to 200-times more selenate concentration, than the MIC value and proved to be stable for selenate resistance as tested by replica plating. The selenate resistant mutants were unable to utilize sulphate as the sole sulphur source. This suggested that mutation occurred in the sulphur assimilation pathway - as in the case of selenate resistant mutants of *Asp. nidulans* (Arst, 1968), *S. cerevisiae* (Breton and Surdin-Kerjan, 1977) and *Asp. fumigatus* (De Lucas et al, 2001).

Complementation analysis of selenate resistant mutants was performed by sexual hybridization of selenate resistant mutants of opposite mating types (h^+ and h^-) having complementing auxotrophic markers. On the basis of genetic complementation, prototrophic hybrids were isolated in every crossing on minimal medium supplemented with cysteine as the sole sulphur source. Sulphate utilization capability of these hybrids, however, was not restored by genetic complementation. This indicated that all the selenate resistant mutants carried the defective mutation in the same gene of the sulphate assimilation pathway.

The *chromate sensitive phenotype (chromate tolerance)* of the mutants did not change compared with wild type strains, which could be the consequence one of the following possibilities: mutation occurred not in the sulphate permease gene, similarly as in the case of sC^- mutants of *Asp. nidulans* or there are more than one chromate permeases operating in *Schiz. pombe.*

Sulphate uptake of two wild type (selenate-sensitive) strains (B 579, 0-82) and their descendent selenate resistant mutants was investigated using a $^{35}SO_4^{2-}$-containing minimal medium. After 1-hour incubation there was a significant difference between the sulphate uptake of the resistant mutants and the wild type strains: mutants were able to take up as much as 54% and 20% of the sulphate assimilated by B 579 and 0-82 wild type strains, respectively. Aiming to check whether sulphate accumulation measured in the case of the resistant mutants was not the consequence of extracellular accumulation of sulphate (i.e. adsorbed by the cell wall), we treated the cells before the sulphate addition with a non-specific metabolic inhibitor, merthiolate. Merthiolate caused complete inhibition of $^{35}SO_4^{2-}$ uptake in 50 µg/ml concentration, both in the wild type and selenate resistant mutant strains studied. This result shows that the decreased sulphate accumulation of the selenate resistant mutants is the consequence merely of the lowered intracellular accumulation and indicates the existence of a permease-mediated sulphate transport in *Schiz. pombe.* The limited decrease of sulphate uptake by the selenate resistant mutants had to allow a considerable growth on sulphate supposing that the activity of the enzymes catalysing the reduction of sulphate to sulphite did not change. Because sulphate did not support the growth of mutants at all, we may conclude that the decreased sulphate uptake was neither the consequence of a "leaky" mutation of the sulphate permease gene, nor that the mutation led to the inactivation of only one of the presumed sulphate permeases. It is more probable that the mutation inactivated one of the genes coding for the sulphate activation (ATP sulphurylase), phosphorylation (APS-kinase) or reduction (PAPS-reductase) and - as the consequence - the intracellularly

accumulated sulphate inhibited its transport as was already demonstrated with *S. cerevisiae* (Logan et al., 1996).

Investigating the *utilization of different inorganic and organic sulphur sources* by selenate resistant mutants, I found that selenate sensitive wild type strains could grow very efficiently in culture media containing 250 µg/ml sulphate. As mentioned before, selenate resistant mutants were unable to propagate cells in the presence of sulphate as the sole sulphur source. Sulphite and thiosulphate proved to be easily assimilable sulphur sources for both the wild type strains and selenate resistant mutants. Organic sulphur sources such as glutathione, cysteine and methionine supported growth of wild type strains, but glutathione and cysteine proved generally better than methionine. This is in accordance with the findings of Brzywczy and Paszewski (1994) who attributed it to the lack of reverse transsulphurylation pathway from methionine to cysteine in *Schiz. pombe*. Surprisingly, all the selenate resistant mutants lost the ability to utilise methionine as the sole sulphur source. This indicates that the main route for the incorporation of the S-content of methionine into the organic compounds is the sulphate assimilation pathway. It is highly probable that the –SH group of methionine is oxidised to sulphate via a degradation route, similarly as it was shown for glutathione in *S. cerevisiae* (Miyake et al., 1999). This sulphate pool is the only sulphur source when methionine is the sulphur supply alone, which cannot be utilised by the selenate resistant mutants in the absence of sulphate to sulphide reduction.

Sulphur source assimilation properties of wild type and selenate resistant mutants were identical not only in the isogenic strains (i.e. L.972, L.975 and their auxotrophic mutants) but also in the case of strains from other sources. This indicates that metabolic routs of sulphate assimilation and methionine utilisation pathway have the same rules within *Schiz. pombe*.

Regarding the enologically important properties of selenate resistant mutant strains we showed that H_2S formation of the tested selenate resistant strains (B 579 Se^R-2 and 0-82 Se^R-2) were not detectable while selenate sensitive wild type cells produced excess sulphide (H_2S) in artificial culture media, must and wine. Malic acid degradation ability remained the same in the mutant strains and fermentation aroma spectrum formed by the mutants were similar to wild type strains as well.

4 NEW SCIENTIFIC RESULTS

(1) *Schiz. pombe* and *Schiz. octosporus* strains with different phenotypic characters have been clustered by dendogram based on RAPD-PCR fingerprints and correlation was found between RAPD clusters and the character of malic acid degradation ability of the strains. RAPD-PCR analysis resulted a very low degree of similarity (30%) between *Schiz. pombe* and *Schiz. octosporus* strains according to the detected differences of their phenotypic characters.

(2) The RFLP analysis of rDNA sequence were applicable for identification at the species level within the genus *Schizosaccharomyces* but did not show any further differences within each of the species. Comparison of restriction sites in the conserved rDNA genes of the three species suggest a closer genetic relatedness between *Schiz. pombe* and *Schiz. octosporus* than between either of them and *Schiz. japonicus.*

(3) Numerous stabil, selenate resistant, sulphate-non-utilizing *Schiz. pombe* mutants have been isolated for the first time, which tolerated 50-200-times more selenate concentration than the MIC value.

(4) All the mutants isolated belong to the same genetic complementation group. Selenate resistant mutant strains B 579 Se^R-2 and 0-82 Se^R-2 tested for sulphate uptake activity showed a decreased intracellular $^{35}SO_4^{2-}$-accumulation which indicated the existence of a permease-mediated transport in *Schiz. pombe.* The mutation inactivated one of the genes encoding for the enzyme ATP sulphurylase, APS kinase or PAPS reductase.

(5) All the selenate resistant mutants lost the ability to utilize methionine indicating that the main route for incorporation of the sulphur atom from methionine is the sulphate assimilation pathway.

(6) The defect in sulphate assimilation pathway had a positive effect on the sulphur containing off-flavour production during fermentation with *Schiz. pombe* strains as hydrogen-sulphide liberated by the tested selenate resistant strains (B 579 Se^R-2 and 0-82 Se^R-2) were not detectable. At the same time malic acid consumption rate and fermentation aroma profile were not affected disadvantageously by the mutation.

5 FURTHER DEVELOPMENT AND UTILIZATION OF THE RESULTS

- For further developement of the results would be worth identificating the inactivated gene, determining the exact locus of the mutation.
- Further study on the incorporation of sulphur content of methionine into the organic compounds in *Schiz. pombe* (methionine to cysteine pathway).
- On the basis of the results I conluded that the tested selenate resistant mutant strains would be applicable in biological malic acid degradation. H_2S production of the mutant strains investigated both by analitical and organoleptical methods was not detectable and mutant strains proved to metabolize malic acid efficiently in must and wine as well.
- Regarding malo-ethanolic deacidification process from technological aspect is the following to study above all: the growing up of required cell mass, as well as quick and costeffective remove of cells from the fermentation media after deacidification has been completed. In this respect it is recommended to test the applicability of immobilized cells, or using up flocculation property of *Schiz. pombe* for lowering the costs. It is highly recommended to improve a suitable strain for commercial wine production by crossing selenate resistant (non-H_2S-forming), good malic-acid-degrading mutants with flocculent *Schiz. pombe* strains.

6 LIST OF PUBLICATIONS

<u>Articles published in journals with impact factors</u>

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