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STUDY OF THE SULPHUR METABOLISM OF METHIONINE-RICH YEASTS

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Abstract

Mutants were produced by mild mutagenic agents such as UV-radiation and nitrite treatment, from yeast strains belonging to different genera. The primary selection has been based on an assumed higher sulphate requirement.

Norleucine has been used as methionine antagonist and the resistance of the mutants was compared to that of the parent strains. Our assays showed that the mutants selected were more sensitive to norleucine as compared to the untreated strains. Thus, the higher methionine content cannot be attributed to homoserine-0-transsuccinase derepression.

Simultaneously with the increased sulphate requirement, the mutants responded to a higher methyl donor concentration also by an enhanced growth.

In methionine enriched mutants also the lipoic acid concentration of the yeast increased in parallel with the increase in the sulphurous amino acid concentration. The concentrations of both components react very sensitively to the aeration intensity applied during fermentation and these begin to fall at the value of 200 mmol $O_2 h^{-1} l^{-1}$.

Sulphur present in sulphate form has been found to be a much better sulphur source than methionine. Applying the latter, a slower cell growth can be observed due to sulphur limitation.

Our experimental results indicate that with the mutants produced, the sulphate reduction step became more efficient and provided an increased methionine content.

Keywords: yeast, sulphur metabolism, methionine, yeast mutants.

Introduction

The deficient methionine and triptophane provision can be taken almost as a people's disease and this, too, contributes to the frequent incidence of various pathological processes (hepatic disease, anaemia). The daily methionine requirement of an adult is about 2.8 g, and this is satisfied only to about 63%, even in case of adequate protein consumption.

In the feeding of monogastric animals, methionine is also an essential amino acid, therefore the amino acid deficiency is overcome by supplementing feeding diets with methionine doses. However, according to feeding trials, the free amino acid shows a more rapid resorption and

thus, only 30–40% of the added methionine is utilized (GEBHARDT, 1977; BURACZEWSKA et al. 1977).

Thus the production of a biomass with increased methionine content constitutes a more effective means for supplementing methionine deficient feed.

KOMATSU and co-workers (1974) successfully produced a mutant rich in methionine from *Candida petrophilum* ATCC 20226. The methionine content of the mutant was about 40% higher as compared to the original strain. OKANASHI and GREGORY (1970) produced, from *Candida tropicalis*, a mutant with a methionine concentration higher by 41%. The increase in methionine concentration was practically entirely the result of the rise of free methionine concentration. KOMATSU and co-workers (1974) reported that during active cell growth, in a shaken culture, the pool methionine concentration was nearly unchanged but decreased in the declining phase of proliferation.

It is worth giving consideration to the proper selection of the parent strain for mutant production as the methionine content of yeasts varies between wide limits, 0.17 – 1.0% (CHIAO and PETERSON, 1953).

ANDERSON and JACKSON (1957) reported the same for bacteria. Among yeasts, mainly the genus *Rhodotorula* was found rich in limiting amino acids.

The key enzyme of methionine synthesis is homoserine-0-transacylase. This is repeatedly de-repressed in feedback resistant mutants. The enzyme is resistant to methionine, S-methyl-methionine, ethionine and norleucine and it is less sensitive to S-adenosyl-methionine than the wild strain (LAWRENCE et al., 1972).

In feedback resistant yeast mutants, pool methionine concentration increases significantly and in numerous cases, the amount of methionine excreted into the surroundings increases as well.

In strains defective in S-adenosyl-methionine synthesis, the enrichment of pool methionine can be observed too, the formed compound being the metabolite of methionine.

The precursor of methionine formation is aspartic acid semialdehyde, similarly to the biological synthesis of threonine, lysine, and isoleucine.

The first specific precursor of methionine is 0-succinyl-homoserine formed through homoserine acylation catalyzed by homoserine-0-trans-succinase (SMITH, 1971). Feedback inhibition is exerted on the enzyme by S-adenosyl-methionine and S-methyl-methionine.

The mutants resistant to ethionine, S-methyl-methionine and norleucine are able to produce methionine in excess and are, therefore, well utilizable for mutant selection.

GREENE et al. (1970) found accumulation of free methionine in the case of low S-adenosyl-methionine synthetase level.

The selection of mutants producing the desired product in greater quantities is a well-proved method in strain improvement work. Another requirement towards the mutants to be considered in our work is that the cells should not excrete the excess amino acid into the culture medium.

UV-irradiation is often applied for the production of mutation though its specific effect is little known. We have more knowledge about the action mechanism of nitrite treatment but there is no possibility to control the site of 'defect' in either of the methods.

Therefore, the cells most favourable for our trials have to be separated from the variety of possible changes with thorough considerations.

In our actual work, manipulation carried out with yeast strains of several genera is reported. As a result, we succeeded in producing new mutants rich in methionine and the role of fermentation parameters could be evaluated as well.

1. Materials and Methods

1.1 Yeast Strains Investigated

For the production of yeast mutants rich in methionine, a new yeast strain belonging to a different genus has been studied. Furthermore, the stability of the mutants B_1 of *Candida guilliermondii* was investigated and the genetical stability of the methionine-rich mutant obtained from *Rhodotorula glutinis* was studied.

Name of the yeast	RNA %	Protein %	Methionine % on dry matter basis
Rhodotorula glutinis CBS 315	–	47.7	0.8
Candida guilliermondii CBS 5256	8.0	46.5	0.5
Candida utilis CBS 5609	6.0	41.1	0.65
Saccharomyces carlsbergensis	7.0	47.1	0.5

1.2 Culture Media Used

In our work, liquid and solid malt media and liquid glucose media – rich/deficient in sulphate – were used.

For the isolation of the mutants and for the production of yeast mutants needed for chemical analyses, yeasts were grown in Petri dishes on a solid enrichment (II) culture medium.

Compositions of the individual culture media:

I. Culture Medium, Deficient in Sulphate

urea	1.93 g/l
$MgSO_4 \cdot 7H_2O$	2.5 mg/l
KH_2PO_4	0.14 g/l
$Na_2HPO_4 \cdot 12H_2O$	0.8 g/l
NaCl	1.0 g/l
glucose	2.0%

II. Sulphate-Rich Culture Medium

$MgSO_4 \cdot 7H_2O$	0.1 g/l
KH_2PO_4	0.14 g/l
$NaHPO_4 \cdot 12H_2O$	1.0 g/l
$(NH_4)_2SO_4$	4.0 g/l
yeast autolysate (10%)	10.0 ml/l
glucose	2.0%

III. Culture Medium

The composition of the culture medium is similar to (II), there is a difference only in the carbon source: 1% glucose + 1% maltose.

All culture media were stabilized with 2% of previously washed and dried agar-agar.

IV. Solid Culture Medium for Growing Saccharomyces Carlsbergensis

malt	3.25 g/l
yeast autolysate	100.00 ml/l
peptone	5.00 g/l
glucose	10.00 g/l
agar-agar	20.00 g/l

V. Synthetic Liquid Culture Medium for the Propagation of Yeast in Shaken Culture

Similar to (II), but contains no yeast autolysate.

Malt-Containing Culture Medium

70 g malt are weighed and added to 1 l water, then autoclaved up to 0.5 bar. After filtering, the refractive index is adjusted to 5 by dilution with distilled water. It is again autoclaved up to bar. For obtaining a solid medium, the ph has to be adjusted to about 5.5–6 and 2% agar-agar is added.

1.3 Determination of Protein Content

The protein content of the biomass was determined by means of the Kjeldahl method with an automatic apparatus, Kjelfoss.

1.4 Determination of Amino Acid Composition

Four our selection work, the methionine content of yeast was determined from hydrochloric acid hydrolysate by means of a microbiological method according to BARTON – WRIGHT (1972) using the *Leuconostoc mesenteroides* P_{60} auxotroph deficiency mutant as test microorganism. Complete amino acid composition was determined in the automatic amino acid analyzer type Labor MIM AMINOCHROM from a sample hydrolyzed for 20 h with 6 mol/l HCl.

1.5 Hydrolysis of the Yeast

50 mg dried and powdered yeast was weighed into a vial, 20 ml 6 mol/l HCl added, the vial sealed and held at 105 ± 2 °C for 24 hours. The vial was opened after cooling to room temperature, the hydrolysate transferred without loss into an evaporating dish and HCl was evaporated in vacuo with several washings. The residue was transferred to a plugged test tube with 3 ml distilled water.

1.6 Determination of Nucleic Acid Content (RNA)

The dried yeast was hydrolyzed with 1 mol/l $HClO_4$ in a water bath of 100 °C for 20 min. The cooled sample was centrifuged and the absorbance of the supernatant measured at 270 nm in a 1 cm quartz cell against 1 mol/l $HClO_4$.

1.7 Method for the Production of Yeast Mutants

The production of yeast mutants was tried with two kinds of mutagenic agents, with nitrite, and with UV-irradiation, respectively. Genetic manipulation was performed according to BOWIEN and SCHLEGEL (1972) applying a nitrite concentration and treatment time established by us.

UV-irradiation was carried out with a germicide lamp manufactured by TUNGSRAM, Budapest.

The pretreatment of yeast cells was similar to that mentioned above, the irradiation time being selected according to the destruction curve mea-

sured. For irradiation, 5 ml of a suspension was weighed into a Petri dish and placed at a 15 cm distance from the lamp.

1.8 Methods Applied for the Selection of Methionine-Rich Mutants

1.8.1 Separation of Methionine-Rich Mutants According to Increased SO_4^{2-} Requirement

Following mutagenic treatment, a 72-hour incubation in a shaken culture in culture medium (II) was applied for the development of mutation and the enrichment of mutants. For the separation of methionine-rich mutants, the colonies spread on malt culture medium (I) were transferred to sulphate-deficient (I) and sulphate-rich (II) synthetic glucose culture media by replica plating technique. The colonies being of greater diameter on sulphate-rich culture medium than in sulphate-deficient conditions were inoculated on to sulphate-rich agar slants. Comparisons were made between the methionine contents of these mutants in relation to the parent strain.

1.8.2 Selection of Mutants Using an Amino Acid Antagonist

The mutant rich in metabolite shows a better tolerance against higher concentrations of anti-metabolites and this phenomenon is often used for selection purposes (SCHERR and RAFELSON, 1962).

In our assays, norleucine was added to culture medium (II) and the beginning and extent of growth were studied in a shaken culture, in comparison with the control without norleucine. For the selection of the mutant, the inhibiting concentration determined for the parent strain was used as indicator.

1.8.3 Mutant Selection Using a Methionine-Homologous Methyl Donor

To our selections, S-methyl methionine sulphonium chloride (vitamin U) was applied in a concentration of 10–60 $\mu g/cm^{-3}$ to culture medium (II) and the colony sizes were studied in comparison with those grown on plain culture medium (II).

The strains studied were inoculated with sterile wooden rodlets on to Petri dishes, to a place previously marked. After the development of colonies, they were transferred with sterile velvet on various culture media with vitamin U content.

1.9 The Yeast Propagation Methods Applied

1.9.1 In Petri Dishes

The yeast streaked on the culture medium stabilized with 2% washed agar was propagated at 32 °C.

1.9.2 In Shaken Culture

Infusion flasks of 500 cm^3, containing 150 ml culture medium were applied for shaken culture. The inoculation was carried out with yeast grown in Petri dishes to 1% yeast concentration. The propagation was carried out in a shaker adjusted to 32 °C.

1.9.3 In Tube Fermentor

A double-walled glass tube of 6 cm inner diameter was used as fermentor. To its bottom opening a gas inlet tube was attached through a G–4 sintered glass filter. The propagation temperature required (32 °C) was assured by circulation of water of appropriate temperature.

The propagations were carried out at aeration intensities of 250, 500, 750, 1000 and 1250 $lh^{-1}l^{-1}$, equivalent to 28, 44, 51, 71 and 84 mmol $O_2l^{-1}h^{-1}$ oxygen transfer rates, respectively.

The pH of the culture medium was held at a constant value by addition of 2.5% NH_4OH solution.

1.9.4 In Laboratory Fermentor

To our trials, two kinds of laboratory fermentors were applied: types BIOFER and CHEMAP. By changing the stirrer revolutions and the rate of air flow, oxygen transfer rates of 60–200 mmol $O_2l^{-1}h^{-1}$ could be adjusted.

During propagation, the pH and temperature were controlled automatically.

1.10 Determination of Oxygen Transfer Rates

The oxygen transfer rates of various propagation devices were determined according to COOPER and MILLER (1944).

1.11 Determination of the Lipoic Acid Content of the Yeast

The lipoic acid content of the yeast was determined according to MUAYAD and co-workers (1983).

1.12 Investigation of Sulphur Uptake of Brewer's Yeast in Labelled Culture Medium

For the trials, sodium sulphate S^{35} and methionine S^{35} were used as sulphur sources.

The liquid culture medium was free from yeast extract and sulphate, and was supplemented with a standard biotin solution (60 μg dm^{-3}).

The nutrient liquor with 2% glucose content was inoculated with 0.5% inoculum. As sulphur source $Na_2^{35}SO_4$ at 1 mCi (37 MBq) and S^{35} methionine, respectively, were used.

Following the inoculation with yeast, sodium sulphate was directly added to the sterile culture medium, then this was agitated for 24 hours at 37 °C with a magnetic stirrer. The yeast suspension was centrifuged (4000 min^{-1}) for 10 min. The precipitate was suspended in 20 cm^3 water and recentrifuged. This procedure was twice repeated using 20 cm^3 ethyl alcohol for the last washing.

The yeast was transferred from the centrifuge tube to a 50 cm^3 flask with alcohol and evaporated to dryness in vacuo. Supernatants obtained by repeated centrifugation were combined, diluted with water to 100 cm^3 and the activity was measured by means of the liquid scintillation method. Hydrolysis was carried out with 25 cm^3 6 mol/l HCl, in an oilbath of 100 – 110 °C temperature, for 5 hours.

The dark brown hydrolysate was clarified with charcoal, washed with water and the clear yellow solution was evaporated in vacuo for the removal of excess HCl. The residue was diluted to 100 cm^3 with water and activity measured with the liquid scintillation system.

The determination of the methionine content of the protein was carried out with radio-TLC method. After dilution to 100 cm^3 with water, the hydrolysate was evaporated in vacuo to dryness and the residue dissolved in 3 cm^3 water. 10 μl of this and along with it – as a standard – methionine S^{35} were applied to Polygram Ionex-25 SA-NA ion exchange thin layer and run in sodium citrate (0.25 mol/l citric acid + 0.25 mol/l sodium hydroxide, ph 3.3) solution. After drying, a radio-chromatogram was made from the layer, in a Berthold LB-2721 instrument.

The part containing methionine was separately scraped from the layer, then the other parts of the layer were scraped together, eluted and their activities measured by the liquid scintillation method.

The results of the two measurements were taken as 100% and from this, the percentage of methionine was calculated.

2. Results

2.1 Production of Methionine-Rich Mutants with Rhodotorula Glutinis CBS 315

2.1.1 Generation of Mutation by Nitrite Treatment

In order to obtain an effective mutation, the influences of nitrite concentration and treatment time on cell destruction were studied. The dependence on nitrite concentration was observed with 1.7, 3.4, 5.1, 6.8, 8.5 and 10.2 mg/cm^{-3} $NaNO_2$ concentrations and 30 min treatment time. The viable germ count was determined by the plate pouring method. The effect of nitrite concentration is shown in *Table 1.*

Table 1
Cell destruction of Rhodotorula glutinis CBS 315 as an effect of $NaNO_2$

$NaNO_2$ (mg cm^{-3})	lg N_0/N
1.7	1
3.4	1
6.8	1
8.5	2
10.2	2

N_0 : living cell number before treatment
N : living cell number after treatment
Treatment time : 30 min

The time dependence of the treatment was determined for the concentration of 3.4 mg/cm^{-3} $NaNO_2$.

Based on our findings, a treatment time of 30 min and concentrations of 3.4, 6.8 and 8.5 mg/cm^{-3} $NaNO_2$ were applied for producing the mutation.

Following enrichment, the colonies streaked on malt culture medium were inoculated by replica plating on culture media (I) and (II). The cells separated according to colony diameters were analysed for methionine. From the colonies separated from the first selection, only six proved to be viable after reinoculation to culture medium (II).

From the well-growing colonies, the quantity needed for methionine and protein determinations was produced in shaken cultures. Based on methionine values determined by the microbial method, only one mutant was found richer in methionine (*Table 2*).

Table 2
Methionine and protein concentrations of the mutants obtained by NO_2 treatment from Rhodotorula glutinis CBS 315

Sign of sample	Methionine[a] (% related to solids)	Protein[b] (% related to solids)
CBS 315	0.80	44.7
315/1	0.65	–
315/2	0.50	–
315/3	0.75	42.7
315/4	0.50	40.3
315/5	0.90	38.8
315/6	0.80	41.1

Values are the mean of the three replicates
[a]standard deviation of the method ±10%
[b]standard deviation of the method ±5%

2.1.2 Generation of Mutation by UV-Irradiation

In preliminary trials, it was found that a 60-min UV-treatment of strain CBS 315 reduced the viable germ count by two orders of magnitude, and therefore this was used for genetical manipulation.

From the colonies grown on culture media (I) and (II), only two colonies could be selected on the basis of colony diameters. Based on methionine and protein values determined from the cell material grown in shaken culture, the strain 315 UV_1 could be taken as a mutant richer in methionine (*Table 3*).

Table 3
Methionine and protein contents of the UV mutants of Rhodotorula glutinis CBS 315

Sign of sample	Methionine[a] (% related to solids)	Protein[b] (% related to solids)
CBS 315	0.80	44.7
315 UV_1	1.00	40.6
315 UV_2	0.50	39.8

Values are the mean of the three replicates
[a]standard deviation of the method ±10%
[b]standard deviation of the method ±5%

2.2 Production of Methionine-Rich Mutants with Saccharomyces Carlsbergensis (Brewer's Yeast)

2.2.1 Generation of Mutation by Nitrite Treatment

For *Saccharomyces carlsbergensis* the nitrite sensitivity of the strain was determined as well. The changes in viable germ count found with 10 and 30 min treatment times and 3.4, 6.8 and 10.2 mg/cm^{-3} $NaNO_2$ concentrations are summarized in *Table 4*.

Table 4
Cell destruction of Saccharomyces carlsbergensis as an effect of $NaNO_2$ treatment (30 min)

$NaNO_2$ concentration (mg cm^{-3})	lg N_0/N
3.4	2
6.8	2–3
10.2	3

Mutant production was performed with 3.4 and 10.2 mg/cm^{-3} $NaNO_2$ concentrations and 30 min treatment time. Separating the colonies exhibiting a higher sulphate requirement based on the methionine determination, it has been found that the four colony forming units all showed a reduction in methionine, protein and RNA concentrations (*Table 5*).

Table 5
Methionine, protein and RNA contents of S. carlsbergensis mutants obtained by NO_2 treatment

Sign of sample	Methionine[a]	Protein[b]	RNA[b]
	(% related to solids)		
S. carlsbergensis	0.5	47.1	7.0
S_{14}	0.3	32.7	4.6
SC_{34}	0.4	31.8	4.0
SC_{1021}	0.25	32.2	5.8
SC_{1022}	0.25	31.7	4.0

Values are the mean of the three replicates
[a] standard deviation of the method ±10%
[b] standard deviation of the method ±5%

2.2.2 Generation of the Mutation with UV-Irradiation

For *S. carlsbergensis* already a 30 min UV-treatment resulted in a viable germ count reduction of 2 orders of magnitude, thus this treatment time was chosen for the production of the mutant.

Based on an increased sulphate requirement, 15 colonies could be selected out of which only 10 proved to be viable after reinoculation. The viable cells were grown on culture medium (II) in a shaken culture, then the methionine content was determined. The results are summarized in *Table 6.*

Table 6
Methionine and protein contents of the UV mutants of S. carlsbergensis

Sign of sample	Methionine[a]	Protein[b]	RNA[b]
	(% related to solids)		
S. carlsbergensis	0.5	47.1	7.0
SC_1	0.65	38.6	7.0
S_2	0.32	42.4	6.2
S_3	0.50	52.8	9.8
S_4	0.80	54.8	11.6
S_5	0.50	49.2	9.7
S_6	0.68	33.6	4.2
S_7	0.25	36.0	5.8
S_8	0.48	54.0	8.0
S_9	0.43	46.0	8.6
S_{10}	0.75	62.2	7.6

Values are the mean of the three replicates
[a]standard deviation of the method ±10%
[b]standard deviation of the method ±5%

Based on methionine values, four mutants were richer in methionine than the initial brewer's yeast. The methionine concentration related to protein was highest in mutant S_6.

2.3 Production of Methionine-Rich Mutants with Candida Utilis CBS 5609

Taking into consideration the nitrite sensitivity of the strain *Candida utilis CBS 5609*, 6.8 and 8.5 mg/cm^3 $NaNO_2$, respectively, and 30 min treatment time were used to obtain a viable germ count reduction by 2 orders of magnitude (*Table 7.*) Seven colonies could be separated based on increased sulphate requirement. The methionine, protein and RNA contents of the yeast grown in shaken culture are given in *Table 7.*

Table 7
Methionine, protein and RNA contents of NO_2 mutants of Candida utilis CBS 5609

Sign of sample	Methionine[a]	Protein[b]	RNA[b]
	(% related to solids)		
C. utilis SBS 5609	0.65	41.1	6.0
FT_3	0.77	42.6	7.0
FT_4	0.75	42.4	7.0
FT_5	0.38	31.6	4.0
FT_6	0.40	43.8	6.0
FT_7	0.50	43.8	6.0
FT_8	0.50	39.1	6.1
FT_9	0.55	33.8	5.0

Values are the mean of the three replicates
[a]standard deviation of the method ±10%
[b]standard deviation of the method ±5%

The results indicate that the mutants FT_3 and FT_4 are richer in methionine, with a satisfactory protein content.

2.4 Production of Methionine-Rich Mutants with the Strain CBS 5256 of Candida Guilliermondii

2.4.1 Generation of the Mutation by $NaNO_2$ Treatment

For the *Candida guilliermondii* strain 5256, of 3.4, 6.8 and 10.2 mg/cm^3 concentrations resulted in D_{10} values of 30, 20 and 10 min, respectively. In order to attain a good mutation effect, the conditions of the treatment were chosen to result in a 99–99.9% destruction in the 10^9 cell/cm^3 yeast suspension (60 min, 6.8 mg/cm^3 $NaNO_2$).

From the methionine-rich mutants, selected after simple mutation, double mutants were produced by repetition. The methionine-rich individuals were selected from the latter. The methionine, protein and RNA contents determined in the yeast mass grown on culture medium (II) are presented in *Table 8.*

2.4.2 Generation of the Mutation with UV-Irradiation

With *Candida guilliermondii* CBS 5256, a 60-min UV-irradiation caused only a 90% cell destruction. From the mutants of increased sulphate requirement none proved to be enriched in methionine.

Table 8
Methionine, protein and RNA contents of the mutants obtained by NO_2 treatment from Candida guilliermondii CBS 5256

Sign of sample	Methionine[a]	Protein[b]	RNA[b]
	(% related to solids)		
C. guilliermondii CBS 5256	0.50	47.2	8.0
1× mutated 1	0.65	47.2	13.4
1× mutated 2	0.70	44.5	7.6
2× mutated from B_1	0.80	45.7	10.0
2× mutated from B_2	0.90	47.5	9.9
2× mutated from C_1	0.75	50.4	9.6
2× mutated from D_2	0.65	42.1	9.6

Values are the mean of the three replicates
[a]standard deviation of the method ±10%
[b]standard deviation of the method ±5%

2.5 Production of Methionine-Rich Mutants with Candida Guilliermondii CBS 812

2.5.1 Generation of the Mutation by $NaNO_2$ Treatment

The *Candida guilliermondii* strain CBS 812 proved to be very sensitive to $NaNO_2$, its D_{10} value (3.4 mg/cm^3) was 15 min. From the 1400 colonies grown after the mutagenic treatment none showed an increased growth on sulphate-rich culture medium.

2.5.2 Generation of the Mutation with UV-Irradiation

The strain CBS 812 seemed to be very sensitive to UV-irradiation.

Its D_{10} value was 2.18 min. Based on an increased sulphate demand, 26 colonies could be separated out of 2080. After the determination of methionine concentration, 4 colonies proved to be enriched in methionine (*Table 9.*)

A triple mutation with UV-irradiation resulted in stable mutants of *Candida guilliermondii* CBS 812. The methionine, protein and nucleic acid contents of the yeast mass grown in tube fermentor are presented in *Table 10.*

Table 9
Methionine, protein and RNA contents of UV-mutants of Candida guilliermondii CBS 812 (propagation in Petri dish)

Sign of sample	Methionine[a]	Protein[b]	RNA[b]
	(% related to solids)		
Candida guilliermondii CBS 812	0.50	44.3	10.8
120 s	0.83	41.2	6.8
180 s	0.80	52.0	5.6
240 s	0.85	43.0	6.3
300 s	0.88	39.0	7.3

Values are the mean of the three replicates
[a]standard deviation of the method ±10%
[b]standard deviation of the method ±5%

Table 10
Methionine, protein and RNA contents of 3x UV-mutants of Candida guilliermondii CBS 812 (propagation in tube fermentor)

Sign of sample	Methionine[a]	Protein[b]	RNA[b]
	(% related to solids)		
CBS 812	0.53	43.9	9.2
S/4	0.71	44.5	6.2
11	0.85	45.1	5.7

[a]standard deviation of the method ±10%
[b]standard deviation of the method ±5%

2.6 Trials Aimed at Improving the Efficiency of Methionine-Rich Mutant Selection

As a first step, increased sulphate requirement was utilized for the separation of methionine-rich mutants from the cells of the parent strain and from the mutants changed in other directions, respectively. The sulphur required for methionine synthesis is taken up by the yeast cells from the sulphate content of the culture medium and utilized, after reduction in several steps to sulphite, then to sulphide, for homocysteine synthesis (*Fig. 1*). Methionine is then formed by methylation of homocysteine.

Taking the increased sulphur utilization as basis, the growth of colonies streaked after mutation was investigated in sulphate-deficient and in sulphate-rich culture media. Comparing the diameters of the colonies obtained by replica plating, the cells assumed to be methionine-rich, were separated.

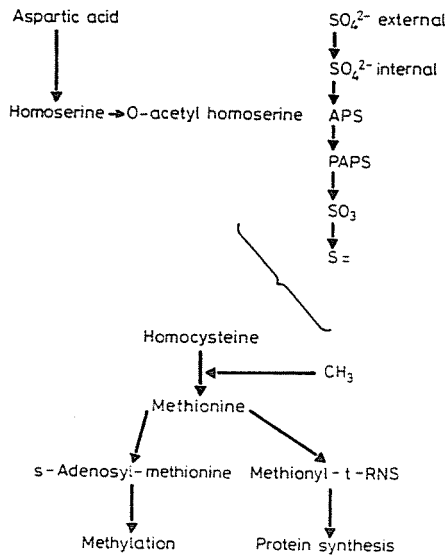

Fig. 1. Sulphur source side of methionine biosynthesis

This selection method proved to be successful. Nevertheless, we were not satisfied by the efficiency of the selection method, as after methionine determination only about 25% of the assumedly favourable cells proved to be really methionine-rich mutants, and some showing negative changes for methionine were detected as well.

To improve our mutant separation work, two versions were studied: utilization of a methionine antagonist and addition of a methionine homologue.

2.6.1 Improvement of Mutant Selection Efficiency by the Use of Norleucine

According to the literature, it is a general experience that the methionine-rich mutant shows a better tolerance towards the antimetabolite and this phenomenon is often used in selection work (SCHERR and RAFELSON, 1962).

From the methionine analogues, ethionine was used most frequently, as this proved to be suitable for the selection of methionine-rich mutants and for the stable maintenance (COLOMBANI et al., 1975; MORZYSKA et al., 1976).

LAWRENCE (1972) observed a resistance to norleucine in methionine-rich mutants.

For the use of norleucine to the selection, the inhibiting concentration related to the parent strain has been determined in the first step.

For the selection of the concentration range, the inhibiting concentrations reported for ethionine were taken into consideration and thus first concentrations of 0.001–0.01% were applied in studying the effect of norleucine on *Candida guilliermondii* CBS 5256.

Shaken colonies were prepared for the assays, and growth was measured after 24 and 48 hours. Norleucine was added in concentrations of 0.001, 0.002, ..., 0.01%, as a total in 10 concentrations. Based on the absorbance and pH changes of the colonies, no inhibiting effect was observed in the starting of growth, nor in its degree (*Table 11*).

Table 11
Determination of the inhibiting norleucine concentration with C. guilliermondii strain CBS 5256

Norleucine concentration (%)	Absorbance of shaken culture 0^h	24^h $\bar{x}_1$	$\pm s$		48^h $\bar{x}_2$	$\pm s$	
0	0.64	0.50	0.05		0.62	0.07	
0.001	0.64	0.58	0.07		0.65	0.06	
0.006	0.64	0.48	0.05	10 times	0.47	0.05	10 times
0.009	0.64	0.59	0.06	diluted	0.51	0.05	diluted
0.01	0.64	0.56	0.07		0.55	0.06	
0.05	0.20	0.10	0.03		1.4	0.05	
0.1	0.20	0.80	0.05		1.4	0.08	
0.2	0.20	0.60	0.07	without	1.3	0.08	without
0.5	0.20	0.40	0.05	dilution	1.4	0.10	dilution
1.0	0.20	0.20	0.03		1.2	0.08	

$\bar{x}_1$, $\bar{x}_2$: mean values of three shaken culture samples
$\pm s$: standard deviation

The inhibiting norleucine concentration was investigated in the range of 0.01–1% for the strains CBS 5256 and CBS 315. Significant inhibition was observed only with 1% norleucine. The starting of growth was delayed for *Rhodotorula glutinis* CBS 315, while for *Candida guilliermondii* CBS 5256 the cell density was only one tenth of those formed at 0.01–0.05%, as demonstrated by data in *Table 11*.

Norleucine sensitivity was compared for *Candida guilliermondii* strain CBS 5256 and mutant B_1. Based on values related to control growth of the untreated cells, a definitely slower growth could be observed for the parent strain and also for the mutant in the presence of 1% norleucine.

The mutant showed a higher sensitivity at this concentration than the parent strain and its inhibition was observed already at 0.5% norleucine (*Table 12*).

Table 12
Comparison of norleucine sensitivities of Candida guilliermondii CBS 5256 and mutant B_1 in shaken culture

Norleucine concentration	Absorbance of Culture CBS 5256			Culture B_1		
	Time (h)					
	0	24	48	0	24	48
0	0.2	0.84	1.4	0.2	0.28	0.59
0.5	0.2	0.44***	1.4	0.2	0.18***	0.19***
1.0	0.2	0.20***	1.2	0.2	0.12***	0.17***

Mean values of 3 parallel measurements
*** Very highly significant at $P \geq 99.9\%$ probability level

2.6.2 Improvement of the Efficiency of Mutant Selection by Use of a Methionine Homologue

Another special requirement of methionine synthesis, besides sulphate, is a methyl donor, therefore it can be assumed that the requirement of the methionine-rich mutant for this should also be higher than that of the initial strain.

S-methyl-methionine sulphonium chloride (vitamin U) is capable of playing the role of a methyl donor and of replacing the methionine in this respect (MCRORIE et al., 1954; HEGEDÜS et al., 1977). In our experiments aimed at the improvement of selection, vitamin U was added to the sulphate-rich culture medium in concentrations of 10–60 $\mu g/cm^3$ and the sizes of the colonies developed were compared to those produced on plain culture medium (II). The diameter of the colonies formed on the control culture medium was the smallest, and with the increase in added vitamin U concentration, the colonies gradually increased.

This could be observed for the original strains (CBS 5256 and CBS 315), as well as for the corresponding mutants (B_1 and CBS 315 UV_1). However, the stimulation on mutants was more expressed.

2.7 Utilization of the Sulphur Sources Sulphate and Methionine, Respectively, by Methionine-Rich Yeast

For our assays, methionine-rich *Saccharomyces carlsbergensis* was used.

2.7.1 SO_4 Utilization of S. Carlsbergensis

$Na^{35}SO_4$ was used in a concentration corresponding to that used in culture medium (II). As a result of our preliminary trials, yeast extract was not applied because with this excess sulphate would have been introduced. This would have decreased the apparent incorporation percentage of the S^{35} isotope derived from S-labelled sodium sulphate. After a propagation of 48 hours using $Na_2^{35}SO_4$ as sulphur source, 269.5 mg yeast was obtained from 100 mg, the resulting plus was 169.5 mg. The remaining nutrient solution and the washing liquids gave a combined activity of 2 294 105 Bq, the activity incorporated in the yeast was 3 887 106 Bq, thus the degree of sulphur incorporation was 94.43%.

The quantitative distribution of sulphur-containing amino acids formed from the sulphate was determined from the radio chromatogram of the hydrolysates obtained after hydrolysis for 2, 5, 10, 15 and 20 h. As it can be seen from *Fig. 2*, each hydrolysate showed three radioactive peaks, one of them for methionine and another for cysteine. The size of the nonidentified third peak decreased with increasing duration of hydrolysis. At the same time, the methionine content increased. The sum of activity percentages represented by the two peaks was constant.

The non-identified peak can be assumed to be methionine-sulphoxide. This is in agreement with the changes with hydrolysis time in the amount of the substance to be identified and with its transformation to methionine, furthermore with the R_f value of methionine-sulphoxide.

2.7.2 Methionine Utilization of S. Carlsbergensis

The culture medium used for the trials contained S-labelled methionine as the sole sulphur source in a quantity similar to the sulphate content of the sulphate culture medium. During propagation of 48 hours, 206 mg of yeast were formed from 100 mg, i.e. the increase was considerably less than the value observed for sulphate medium.

Based on the combined activity of the nutrient liquid and washing solution, and on the labelled sulphur detectable in yeast, only 45% was incorporated from the methionine present in the culture medium. This incorporation ratio was lower than that found with the sulphate medium even when taking into consideration that the cell growth was less in the methionine containing medium. According to the radiochromatogram of the yeast hydrolysate, 45% of the uptaken activity was present in the form of methionine in yeast. This proves that the proportion of sulphur-containing

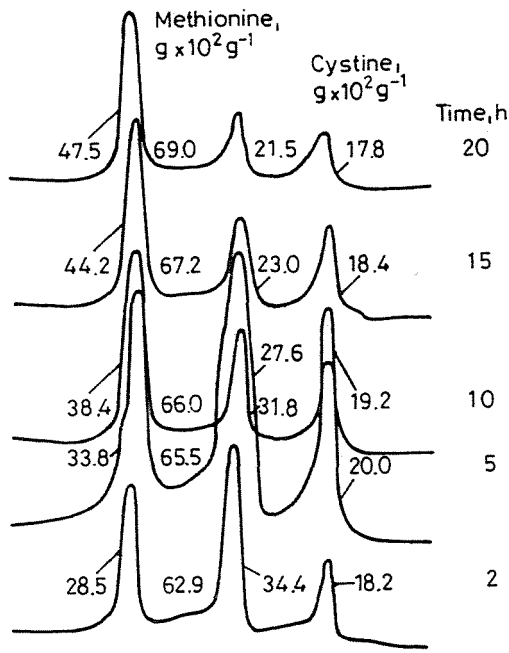

Fig. 2. Radiogram of the hydrolysis product of *S. carlsbergensis.* (Quantitative evaluation of the radiogram can be seen in *Table 13*)

amino acids of *S. carlsbergensis* does not depend on the fact whether the sulphur source was sulphate or methionine.

2.8 The Effect of Oxygen Transfer Rate in Yeast Propagation on Methionine and Lipoic Acid Concentrations

In our work we found that the cell mass grown in Petri dishes showed the highest methionine content, for parent strains as well as for methionine-rich mutants. The methionine concentration of cells grown in shaken cultures or tube fermentor is considerably lower (*Table 13*).

Within the range of oxygen transfer rates of 28–84 mmol $O_2 l^{-1} h^{-1}$ up to 58 mmol $l^{-1} h^{-1}$ the methionine concentration was increased. However, with 84 mmol $l^{-1} h^{-1}$ it showed already a decrease as compared to the highest value measured for the mutants *Candida guilliermondii* 11 and 514, as it is to be seen in *Figs. 3* and *4*.

In laboratory fermentors provided with an agitator for increased oxygen supply, the methionine content of the biomass formed showed the re-

Table 13
Investigation of ^{35}S incorporation in the hydrolysate of S. carlsbergensis grown on $Na_2^{35}SO_4$ culture medium

Hydrolysis time (h)	Methionine (%)	Non-identified peak (%)	Cysteine (%)	Methionine + identified peak (%)
2	28.5	34.4	18.2	62.9
5	33.8	31.8	20.0	65.5
10	38.4	27.8	19.2	66.0
15	44.2	23.0	18.4	67.2
20	47.5	21.5	17.8	69

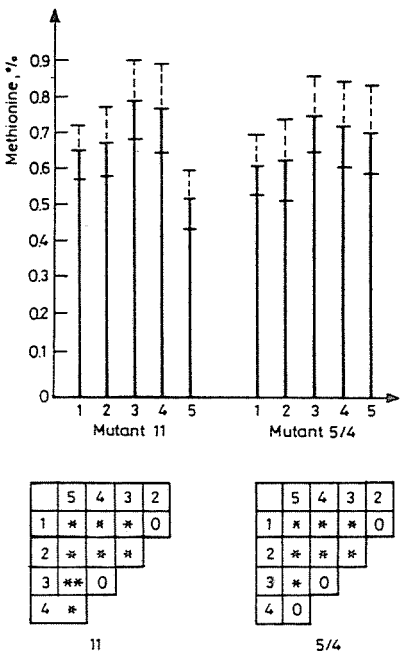

Fig. 3. Influence of oxygen transfer rate on the methionine content of mutants 11 and 5/4 (propagation in tube fermentor). 1, 2, 3, 4, 5 oxygen transfer rates = 28, 44, 58, 71, 84 mmol $O_2 l^{-1} h^{-1}$. Methionine content % of dry material. *Significant at $P \geq 95\%$ probability level; **highly significant at $P \geq 99\%$ probability level; $\emptyset$ no significant difference

sults given in *Fig. 8* for parent and mutant strains, with 70 and 220 mmol $O_2 l^{-1} h^{-1}$ oxygen transfer rates, respectively.

The methionine content was found to decrease with the intensification of the aeration.

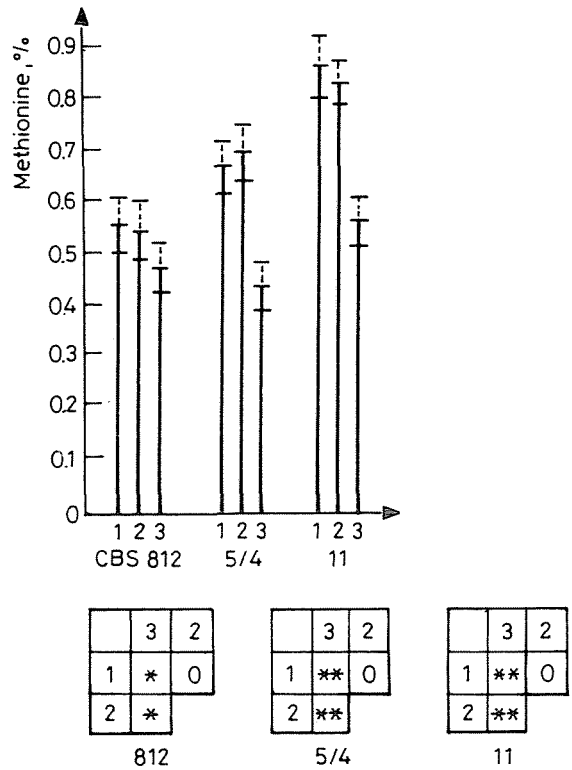

Fig. 4. Selection of methionine-rich yeasts on methyl donor medium. 1, 2, 3: oxygen transfer rates = 70, 150, 200 mmol $O_2 l^{-1} h^{-1}$. Methionine content % of dry material. *Significant at $P \geq 90\%$ probability level; **highly significant at $P \geq 99\%$ probability level; ∅ no significant difference

With the increase in oxygen transfer rate the lipoic acid content of yeast cells underwent a change similar as shown by the data summarized in *Table 14*.

3. Discussion

3.1 Production of Methionine-Rich Mutants with Gentle Mutagenic Agents

Our results prove that even gentle mutagenes can be effectively used for the production of methionine-rich yeast mutants, similarly to the powerful mutagenes applied.

With multiple mutation, the methionine content can be nearly doubled as compared to the initial value.

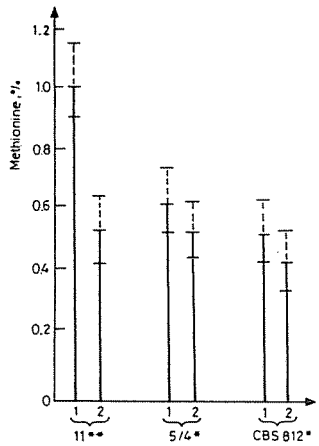

Fig. 5. Influence of oxygen transfer rate on the methionine content of *Candida guilliermondii* 812 and mutants 11 and 5/4 (propagation in laboratory fermentor type Chemap). 1,2: oxygen transfer rate = 90, 200 mmol $O_2 l^{-1} h^{-1}$. *Significant at $P \geq 95\%$ probability level; **highly significant at $P \geq 99\%$ probability level

Table 14
Methionine content of the mutant B_1 of Candida guilliermondii CBS 5256 in cultures with different O_2 transfer rates

Petri dish	1	Shaken culture	Column fermentor	Laboratory fermentors	
				Biofer	Chemap
		2	3	4	5
Methionine related to solids (%)	0.80 ±0.07	0.70 ±0.08	0.62 ±0.06	0.62 ±0.06	0.52 ±0.05

Test of significance

	1	2	3	4
5	***	***	***	***
4	***	***	Ø	
3	***	***		
2	***			

*** Very highly significant at $P \geq 99.9\%$ probability level
Ø no significant difference

Mutant Selection

The methionine-rich mutants produced with gentle mutagenes have an enhanced sulphate requirement and this can be used successfully for their selection as well.

In contrast to the results of LAWRENCE (1972) we found that the mutants are more sensitive to norleucine than the parent strains. This phenomenon indicates that in the methionine-rich mutants produced, the increase in the concentration of sulphur-containing amino acids cannot be attributed to the derepression of homoserine-0-transsuccinase.

The increased methyl donor requirement, i.e. the addition of the methionine homologue vitamin U resulted in an increased colony diameter as compared to the control. This proves that the utilization in the formation of cell material, of the better sulphur supply in sulphate-rich media is accompanied by a higher methyl donor requirement. With the mutant of higher methionine content, this appears to a higher degree.

The results of investigations on the utilization of the sulphur source by methionine-rich yeasts prove that these yeasts do not directly incorporate methionine. First this is oxidized to sulphate and then – through reductive pathways – to methionine.

Thus, growth conditions limited with respect to sulphur are created as compared to sulphate containing culture medium, resulting in a slighter cell multiplication.

The ratios of sulphur-containing amino acids do not depend on the kind of sulphur source, i.e. sulphate or methionine but the absolute quantity for methionine containing medium is about 23% lower for sulphate containing medium as calculated from the percentages of cell mass and sulphur incorporation ($163 : 106 = 94.43 : x = 58$, in contrast to 45%, if the former corresponds to 100% methionine and the latter to 77%).

Effect of Oxygen Transfer Rate on Methionine and Lipoic Acid Contents of Yeast

Taking into consideration the values used in general for yeast propagation, the methionine and lipoic acid concentrations of the yeast begin to decrease for the parent strains as well as for the mutants already at the low value of 200 mmol $O_2 l^{-1} h^{-1}$.

This phenomenon can be attributed to the utilization of sulphur originating from sulphate that is transformed through several reductive steps to sulphide (HILTE et al., 1959). In this process, lipoic acid is the prostethic group in sulphate reductase involved in the transformation of SO_4^{2-} to SO_3^{3-}.

Table 15
Effect of oxygen transfer rate on the methionine and lipoic acid contents of Candida guilliermondii 812 and mutant 11

Strain	Transfer rates (O_2 mmol $l^{-1}h^{-1}$)	Methionine (g per 100 g)		Lipoic acid (mg per 100 g)	
		$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$
CBS 812	200	0.45	0.03	0.35	0.03
	50	0.60	0.04**	0.42	0.05*
11	200	0.75	0.08	0.35	0.04
	50	1.00	0.10**	0.47	0.05*

* Significant at $P \geq 95\%$ probability level
** Highly significant at $P \geq 99\%$ probability level

4. Conclusions

The methionine-rich mutants produced with gentle mutagenic agents such as nitrite and UV-irradiation have an increased sulphate and methyl donor requirement.

They show a higher sensitivity to norleucine than the parent strain, thus the increased methionine concentration cannot be attributed to feed-back resistance to the de-repression of homoserine-0-transsuccinase.

The increased lipoic acid content of the mutants and the sensitivity to the oxygen supply of the medium result in lower methionine and lipoic acid concentrations at higher O_2 transfer rates. This indicates that for the mutants produced the sulphate reduction step became more effective and this resulted in an increased methionine content.

At the same time it is noteworthy that, in contrast to the mutants produced with powerful mutagenes, in our assays the increase in methionine concentration occurred not in the higher concentration of free amino acids but in methionine in peptide-linkage.

Acknowledgement

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