PhD dissertation thesis

IMPROVEMENT OF *KLUYVEROMYCES MARXIANUS* YEAST STRAINS FOR BIOETHANOL PRODUCTION

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1. Historical background and objectives of the doctoral dissertation

Utilization of renewable energy is getting more and more important in our lives by the exhaustion of fossil energy sources and by economical perspectives and environmental protection coming to the forefront.

Bioethanol is one of the renewable energy sources, and the use of it may reduce pollution produced by the combustion of fossil energy sources. Production based on agricultural products and by-products reached a technological level that can be competitive on the energy market.

Bioethanol can be produced from different kinds of feedstock, for example sugar-containing (sugar cane, beetroot, etc.) or starch-containing (corn, wheat, etc.) crops or cellulose containing woody plants. The cellulose-based production relies not only on crops but also on agricultural residues (grape residues, corn stover etc.). The usage of these feedstock is free of extra cost and environmental impact.

A profitable way to produce ethanol is the application of thermotolerant yeasts. *Kluyveromyces marxianus* is a yeast species with outstanding thermotolerance. *K. marxianus* yeast strains have great future potential due to their wide substrate spectra, heat tolerance and fast growth rate (Singh *et al.*, 1998b). These strains are able to synthesize bioethanol according to Simultaneous Saccharification and Fermentation (SSF) experiments and immobilisation techniques as well, which indicates possible industrial value (Love *et al.*, 1998). Other literature data refer to the heat tolerance of this strain as being able to be further increased to the temperature level of the starch hydrolysis (Fonseca *et al.*, 2008)

The aim of my PhD project was to develop a *K. marxianus* strain in order to produce the highest amount of ethanol possible at a temperature higher than that applied currently in industrial production. According to the literature data the *K. marxianus* IMB3 strain has one of the best heat tolerance (Banat és Marchant, 1995), it is able to grow at 52°C degree and produce ethanol at 50°C degree. We would have liked to study the IMB3 strain but, it is already in industrial use and so it is only available for the research group which works with it. Thus we were working with the CBS712 strain according to the recommendation of the NCYC. The advantage of this strain is that the 20 % of its genome has already sequenced (Llorente *et al*., 2000) and its heat tolerance is remarkable.

During my PhD work my implemented aims were as follows:

- Optimalization of the fermentation parameters of *K. marxianus* CBS712 strain (pH, the amount of inoculum, oxigen demand, starting glucose concentration).
- Investigation and increase of its heat and general stress tolerances.
- Isolation of an increased heat tolerance mutant, which comes from continuous passageing of the parental strain at increased temperature.
- Taxonomical, morphological and physiological investigations of the parental and the mutant strains.

2. Materials and methods

2.1. Maintenance of *K. marxianus* CBS712 (NCYC 2791) and the E1 mutant strains

K. marxianus CBS712 (NCYC 2791) was bought from the NCYC (National Collection of Yeast Culture), and the *K. marxianus* E1 thermotolerant mutant was developed from this strain. These strains were maintained routinely on YPD and YM agar plates incubated at 30 °C.

The YPD agar:

- 1% yeast extract (BBL™),
- 2% peptone (Difco),
- 2% glucose,
- 2% agar.

The YM agar:

- 0,3% yeast extract (BBL™),
- 0,3% maláta kivonat,
- 0,5% peptone (Difco),
- 1% glucose,
- 2% agar.

The pH of the YPD and YM agar plates was set to 5.5. The maintenance of these strains for long time was at -70˚C in YM broth supplemented with 20 % glycerin.

2.2. Development and identification of the thermotolerant *K. marxianus* E1 mutant strain

K. marxianus CBS712 colonies were inoculated into 5 ml aliquots of YPD broth, and were cultivated with shaking at 48 ˚C and 3 Hz shaking frequency for 2 days. Cultures displaying significant growth were transferred into fresh YPD broth and were cultivated further at 48 ˚C for 2 days. This procedure was repeated 10 times, and the best-growing culture was spread out on YPD agar. One colony (E1) was chosen for further investigations.

The E1 mutant was identified as *K. marxianus* following the methods and criteria of Barnett *et al.* (1990).

2.3. Stability investigation of the thermotolerant phenotype

The stability of the thermotolerant phenotype was also demonstrated by culturing the E1 mutant in YPD broth under non-selective condition (30 ˚C) for 2 days, and transferring it into fresh medium. This procedure was repeated 10 times, and the growth of the strain was checked at 48 ˚C.

2.4. Growth of the *K. marxianus* CBS712 and the E1 mutant strains

The components and the pH of the YPD broth were the same as the YPD agar plates except. Besides the growth was tested in MYFM broth (Hack and Marchant, 1998b) supplemented with appropriate amount of glucose.

MYFM broth:

- 3 g l^{-1} yeast extract (BBL^{TM}),
- 2 g l^{-1} peptone (Difco),
- 2 g l^{-1} KH_2PO_4,
- 2 g l^{-1} $(NH_4)_2SO_4$,
- 1 g l^{-1} $MgSO_4$* $7H_2O$,
- 1 g l^{-1} $MgSO_4$* H_2O.

Overnight cultures (10 ml YPD broth) were inoculated with individual colonies from fresh YPD or MYFM agar plates and were cultivated at 30 °C. The growth kinetics of the *K. marxianus* CBS712 and E1 strains were characterized by measuring the changes on OD_{600} in YPD or MYFM broths (100 ml) at 30, 35, 40, 45, 46, 47 or 48 ºC and 3 Hz shaking frequency for 24 h. The starting OD_{600} was set to 0.1 with overnight cultures.

2.5. Measurement and optimization of ethanol production

To optimize ethanol yields, *K. marxianus* strains were cultivated in 250 ml Erlenmeyer flasks containing 100 ml MYFM broth. The selected starting glucose concentrations were 0.50, 0.71, 0.91, 1.11, 1.31 or 1.51 mol l^{-1} and the fermentation temperature was set to 35, 37, 39, 41, 43, 45, 47 or 48 ˚C. Samples were taken at 16, 24 and 38 h incubation times.

Ethanol concentrations were measured by gas chromatography using an HP 5890 series II gas chromatograph equipped with automata sampler, split injection (1:10, 225 ^{o}C), HP 5 capillary column (35 ^{o}C, nitrogen carrier gas flow rate 1 ml min^{-1}), and ChemStation software for data handling. As an internal standard, 0.166 mol l^{-1} isopropanol was used, and the linearity of the calibration curve was confirmed for ethanol in the concentration range of 0.09-0.86 mol l^{-1} (r= 0.9997).

2.6. Measurement of residual glucose concentration

Residual glucose concentration was measured by thin layer chromatography. Samples (0.5 µl) were applied to HPTLC silica gel 60 plates (20x20 cm, Merck), which were developed in dichloromethane:methanol:water (30:25:6) solution, were dried and sprayed with 10 v/v % sulfuric acid dissolved in absolute ethanol. To visualize the spots, the plates were dried and heated up to 120 ˚C for 5 min. Spot areas were quantified by the CPAtlas 1.0 program (developed by Dr. István Lázár, http://sites.google.com/site/lazaristvan99/) using calibration curves (0.03-0.3 mol l^{-1} glucose).

2.7. The conversion rate (ethanol yield)

Ethanol yield (%) was calculated according to the following formula: [(A/(B-C))/2]*100 where A, B and C stand for the produced ethanol (mol), the starting glucose content (mol), and the residual glucose content (mol), respectively, and 2 mol ethanol (mol glucose)$^{-1}$ is the theoretical ethanol yield.

2.8. Determination of GSH and GSSG concentrations

For reduced (GSH) and oxidized (GSSG) glutathione determinations, the yeast cells were cultured according to chapter 2.4.. The yeast cells were separated from 50 ml aliquots of the cultures by centrifugation (4000 g, 10 min, 4 °C) and the cells were resuspended in 4 ml of ice-cold 5 % (v/v) 5'-sulfosalicylic acid by vigorous mixing. After centrifugation (10000 g, 10 min, 4°C), the supernatants were neutralized with triethanolamine (Emri *et al.*, 1997).

The intracellular GSSG concentrations were determined according to the Anderson's rate assay (1985). The compounds in reaction mixture were: 115 mmol l^{-1} sodium-phosphate buffer (pH: 7,5), 50 mmol l^{-1} EDTA, 0,6 mmol l^{-1} 5,5'-ditio-*bis*(2-nitrobenzoe acid) (DTNB), 0,2 mmol l^{-1} NADPH, 1,5 kU l^{-1} GR (glutathione reductase), 10 % (v/v) sample. The change in reduced DTNB was quantified by measuring optical densities at 412 nm. The GSH contents were treated by 2-vinilpiridine (185 mmol l^{-1}, 1 hour, pH 6,0-7,0).

The intracellular GSH concentration was determined according to the Anderson (1985). We measured the whole gluthatione content (GSH+GSSG) without 2-vinilpiridine treatment. The GSH content was calculated from the GSSG and the whole glutathione contents.

2.9. Determination of the trehalose content

Trehalose contents were determined in MYFM cultures (30 ^{o}C, 3 Hz) with temperature shifts to 47 ^{o}C at 8 h cultivation times. Samples were taken at 0, 30, 60, 90, 180 min after the temperature shifts. Trehalose was extracted according to Ferreira *et al.* (1997), and the trehalose contents of the extracts were determined by HPLC using a Hewlett-Packard 1090 Series II liquid chromatograph equipped with a diode array detector and automatic sampler. Compounds were separated on a HILIC column (YMC Polyamine II, 250x4.6 mm, 5 μm) with acetonitrile:water (60:40) mobile phase (flow rate of 1 ml min^{-1}, 25 ^{o}C). Optical densities recorded at wavelengths λ=190 and 200 nm were analyzed by ChemStation software. Trehalose was quantified using α,α-D-trehalose-dihydrate standard (1-100 mg ml^{-1}; Senn Chemicals).

2.10. Determination of ethanol, oxidative stress and osmotic stress tolerance

In stress tolerance studies, MYFM broth (3 g l^{-1} yeast extract, 2 g l^{-1} peptone, 2 g l^{-1} KH_2PO_4, 2 g l^{-1} $(NH_4)_2SO_4$, 1 g l^{-1} $MgSO_4$ 7 H_2O; pH=5.0; Hack *et al.*, 1998) cultures were pre-cultivated at either 37 or 45 ˚C, and were used to inoculate 100 ml aliquots of MYFM broth. Selected cultures were supplemented with 0.65, 1.09 or 1.38 mol l^{-1} ethanol, 1.0, 1.25, 1.5 or 2.0 mol l^{-1} NaCl or KCl or with 5, 7.5, or 10 mmol l^{-1} H_2O_2 as required, and were incubated at 37 ˚C for 24 h. The starting OD_{600} values were always set to 0.1, and microbial growths were characterized by increases in the optical density (OD_{600}) of the fermentation broths.

2.11. Response Surface Methodology (RSM)

Optimal conditions (starting glucose concentration, temperature and cultivation time) for maximal ethanol production and maximal differences in the ethanol production between the CBS712 and E1 strains were also determined. Ethanol concentrations ($c_{ethanol}$; mol l^{-1}) were transformed to $\log(c_{ethanol} + 0.1)$ values. Transformed ethanol concentrations were fitted using second order local polynomial regression (LOESS) with case weights ($c_{ethanol}$ + 0.1) mol l^{-1} and 7 ºC temperature difference corresponding to 1 mol l^{-1} glucose concentration difference, while fitting to time was global using the R statistical software (http://www.R-project.org).

3. Results

3.1. Development and identification of the *K. marxianus* thermotolerant E1 mutant strain

A *Kluyveromyces marxianus* thermotolerant strain (E1) was developed from the CBS712 strain by continuous cultivation at increasing temperature as described in the Materials and methods section. The mutant strain grew similarly to the parental at 45 °C but it was able to grow even at 48 °C (Fig. 1). This property of the mutant was stable even after transferring it 10 times under non selective conditions (30 °C).

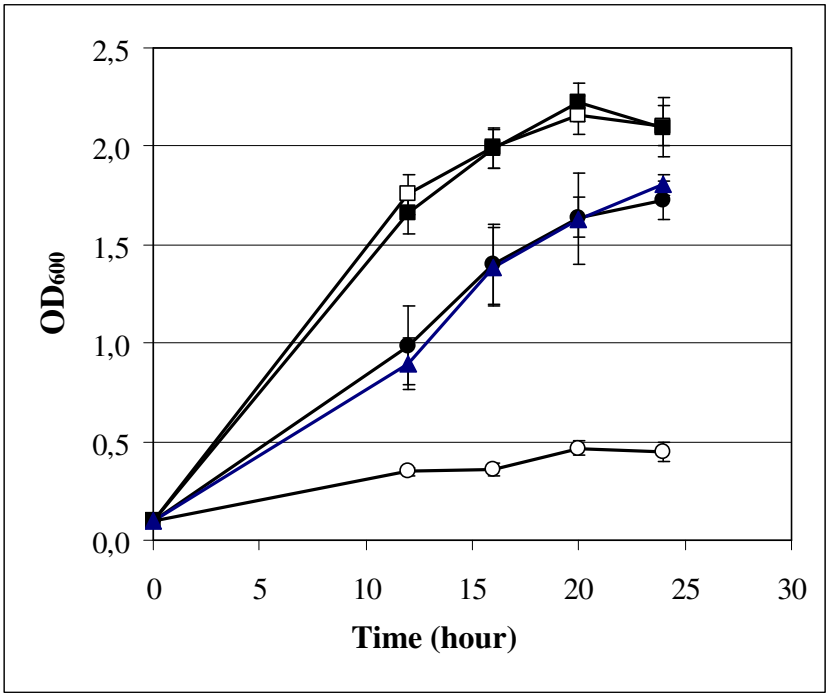

Figure 1. Growth of the *Kluyveromyces marxianus* CBS712 parental (open symbols) and E1 mutant (closed symbols) strains at 45 °C (□, ■) and 48 °C (○,●) in MYFM medium and growth of the E1 mutant at 48 °C after transferring it 10 times under non selective conditions (30 °C) (▲). Mean ± S.D. values calculated from three independent experiments are presented.

3.2. Characterisation of the *K. marxianus* CBS712 parental and E1 mutant strains

To determine the optimal ethanol production of the E1 mutant strain and to compare these results to the features of the parental strain we chose the RSM method (Response Surface Methodology).

To carry out RSM, *K. marxianus* strains were cultivated in 250 ml Erlenmeyer flasks containing 100 ml MYFM broth. The selected starting glucose concentrations were 0.50, 0.71, 0.91, 1.11, 1.31 or 1.51 mol l^{-1} and the fermentation temperature was set to 35, 37, 39, 41, 43, 45, 47 or 48 ˚C. Samples were taken at 16, 24 and 38 h incubation times.

We defined the conversion rate (ethanol yield), which indicated the efficiency of the ethanol production. For the calculations, we needed the amount of residual glucose concentration, which were determined by Thin Layer Chromatogaphy.

As demonstrated by RSM (Figure 2.), maximal ethanol concentrations were at similar values for the parental and mutant strains, namely at 1.03 mol l^{-1} starting glucose concentration, 44.2 °C incubation temperature and at 30 h incubation time for the parental strain, and at 44.8 °C for 31 h with a starting glucose concentration of 1.09 mol l^{-1} in mutant yeast cultures (Figure 2; Tables 1). The regression surfaces also suggested that under optimal conditions the ethanol production of the strains were fairly similar, however at higher temperatures the mutant produced more ethanol than the parental strain (Figure 2). The most significant difference in ethanol yields were expected in cultures containing 1.09 mol l^{-1} glucose and incubated at 47.2 °C for 35 h.

- The ethanol and biomass productions of the mutant and parental strains were quite similar {1.0±0.1 *vs.* 1.1±0.1 mol l^{-1} (4.6±0.5 *vs.* 5.1±0.5 w/v %) and 42±9 *vs.* 51±9 g l^{-1}, respectively)} when cultivated under nearly optimal producing conditions {44 °C, 1.06 mol l^{-1} (19.1 w/v %) glucose}.
- The E1 mutant produced more ethanol and biomass when the incubation temperature was elevated to 47 °C at 1.09 mol l^{-1} (19.6 w/v %) starting glucose concentration {0.6±0.1 *vs.* 0.3±0.1 mol l^{-1} (2.8±0.5 *vs.* 1.4±0.5 w/v %) and 42±8 *vs.* 29±7 g l^{-1}, respectively)}.

	Ethanol ($mol\ l^{-1}$)		**Residual glucose ($mol\ l^{-1}$)**		**Biomass ($g\ l^{-1}$)**		**Ethanol yield [b] (%)**	
	Parental strain	E1 mutant	Parental strain	E1 mutant	Parental strain	E1 mutant	Parental strain	E1 mutant
Optimal conditions	1.1 ± 0.1	1.0 ± 0.1	0.24 ± 0.04	0.26 ± 0.04	51 ± 9	42 ± 9	67 ± 6	63 ± 6
47 ºC	0.3 ± 0.1[d]	0.6 ± 0.1[c,d]	0.54 ± 0.06[d]	0.36 ± 0.04[c,d]	29 ± 7[d]	42 ± 8[c]	27 ± 9[d]	41 ± 7[c,d]

Table 1. Comparison of the ethanol productions of the *Kluyveromyces marxianus* CBS712 parental and E1 mutant strains under optimal conditions and at high temperature.[a]

[a] - Under optimal ethanol production conditions the starting glucose concentration and the incubation temperature were set to 1.06 mol l^{-1} and 44 °C, respectively. Under high temperature conditions, the starting glucose concentration was 1.09 mol l^{-1} and the cultures were incubated at 47 °C. Samples were taken and analyzed at 30 h (optimal condition) or 35 h (high temperature condition) incubation times. All data shown in this Table represent mean ± S.D. values calculated from three independent experiments. *p* values were calculated using the Student's *t*-test.

[b] - The ethanol yield was calculated according to the following formula: [(A/(B-C))/2]*100 where A, B and C stand for the produced ethanol (mol), the starting glucose content (mol), and the residual glucose content (mol), respectively, and 2 mol ethanol (mol glucose)$^{-1}$ is the theoretical ethanol yield.

[c] - Significant ($p < 0.05$) differences between the parental and mutant strains.

[d] - Significant ($p < 0.05$) differences between the optimal (44 ^{o}C) and high temperature (47 ^{o}C) growth conditions.

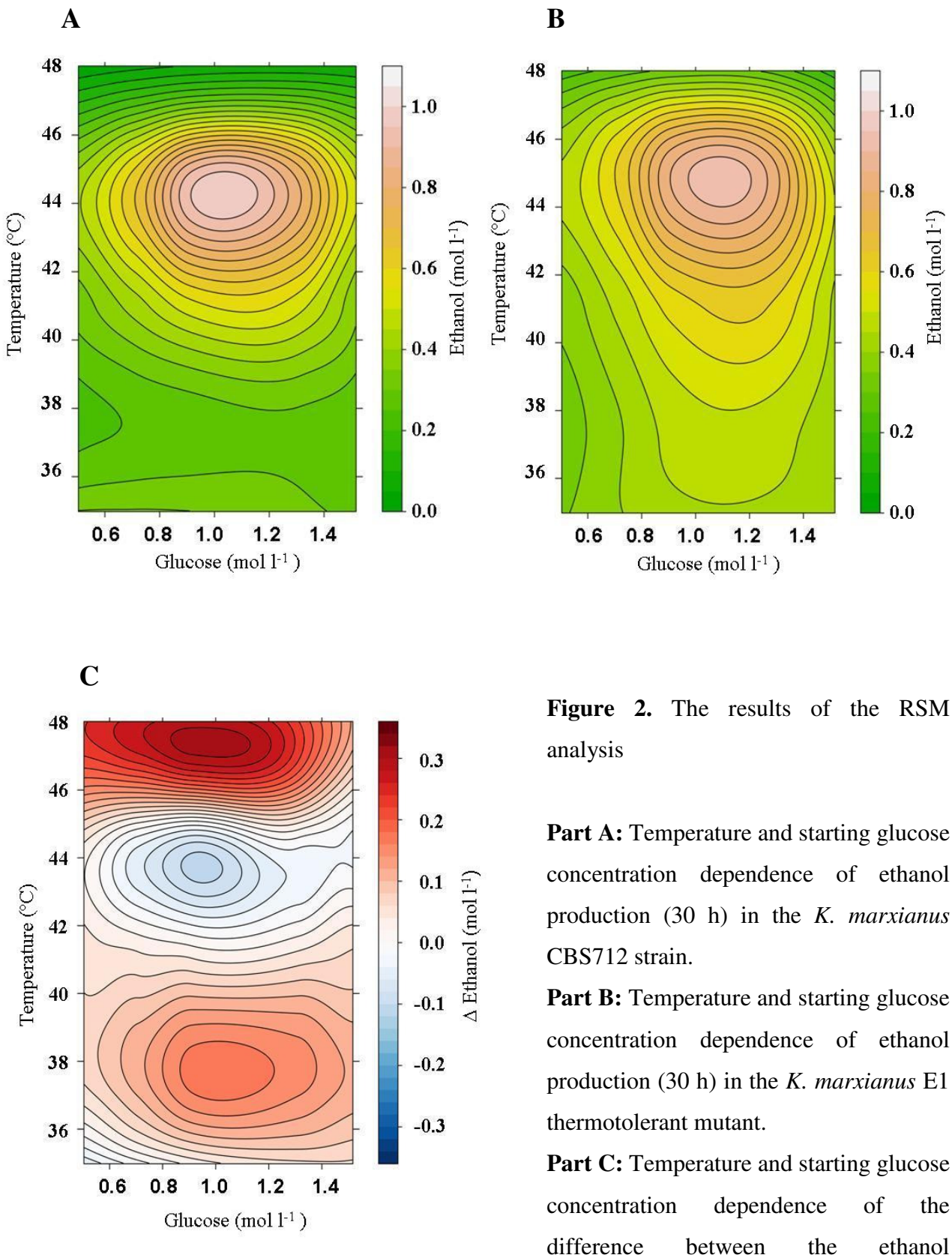

Figure 2. The results of the RSM analysis

Part A: Temperature and starting glucose concentration dependence of ethanol production (30 h) in the *K. marxianus* CBS712 strain.

Part B: Temperature and starting glucose concentration dependence of ethanol production (30 h) in the *K. marxianus* E1 thermotolerant mutant.

Part C: Temperature and starting glucose concentration dependence of the difference between the ethanol productions (Δ Ethanol) of the mutant and parental strain.

The maximum ethanol yields of the CBS712 and E1 strains were comparable to those of the IMB3 {4.3 w/v % (0.93 mol l^{-1}) at 45 ^{o}C, on molasses medium with initial reducing sugar concentration of 16 w/v %; Banat *et al.* (1992)} and the DMKU3-1042 {4.93 w/v % (1.07 mol l^{-1}) at 45 ^{o}C, on sugar cane juice supplemented with sucrose to 8 w/v % sugars; Limtong *et al.* (2007)} strains but a higher ethanol yield {5.9 w/v %, (1.28 mol l^{-1})} with a high, 87 % ethanol yield was recorded for the IMB3 strain fermenting 14.0 w/v % glucose at 45 ^{o}C (Banat *et al.*, 1992).

The appropriate ethanol yields for the CBS712 and E1 strains were lower, 67±6 % and 63±6 %, respectively. At higher temperatures, the E1 mutant was superior to the CBS712 parental strain however the ethanol yields declined spectacularly even within the E1 strain (41±7 %, 47 ^{o}C).

According to the results the industrial SSF technology could be more efficient with application of the *K. marxianus* E1 mutant strain, however to confirm this we have to carry out further investigations.

3.3. Physiologycal studies of the *K. marxianus* E1 mutant strain

We carried out physiological investigations with the E1 mutant in order to understand the physiological background of its thermotolerant phenotype. At first, we studied the intracellular glutathione (GSH) and glutathione disulphide (GSSG) concentration of the parental and the mutant cells, but we did not find any differences between the two strains in this regard.

The increased thermotolerance of the E1 mutant was attributed to its significantly elevated trehalose production under either thermal stress or unstressed conditions (Table 2).

Incubation time (min)	Trehalose content {mg (g dry cell mass)$^{-1}$}			
	30 °C		47 °C	
	Parental strain	E1 mutant	Parental strain	E1 mutant
0	12 ± 2	30 ± 3[b]	11 ± 2	30 ± 2[b]
30	11 ± 2	34 ± 2[b]	9 ± 2	31 ± 2[b]
60	9 ± 2	30 ± 2[b]	11 ± 1	35 ± 3[b,c]
90	9 ± 2	32 ± 3[b]	37 ± 3[c]	57 ± 2[b,c]
180	14 ± 3	28 ± 2[b]	35 ± 2[c]	59 ± 2[b,c]

Table 2. Effect of thermal stress on the trehalose contents of the *Kluyveromyces marxianus* CBS712 parental and E1 mutant strains. [a]

[a] - All data shown here represent mean ± S.D. values calculated from three independent experiments. *p* values were calculated using the Student's *t*-test.

[b] - Significant ($p<0.05$) differences between the trehalose contents of the parental and mutant strains.

[c] - Significant ($p<0.05$) increases in the trehalose contents when compared to 0 h data.

Interestingly, Nwaka *et al.* (1994) did not find any strong correlation between trehalose accumulation and increased thermotolerance in the baker's yeast *Saccharomyces cerevisiae* and, consequently, suggested that the increased trehalose synthesis is only the first rapid response to heat stress and the accumulation of heat-shock proteins is needed for a long-term protection. We found no significant differences in the SDS PAGE protein band intensities of the E1 mutant when the strain was cultivated at 45 or 48 ^{o}C.

Unexpectedly, the increased trehalose content was accompanied by an enhanced sensitivity to NaCl, ethanol, KCl and H_2O_2 stress (Table 3).

		OD_{600} (%)[a]	
		37 °C inoculum	45 °C inoculum
K. marxianus CBS712	1 M KCl	87 ± 7	90 ± 1
	1.25 M KCl	77 ± 7	84 ± 2
	1.5 M KCl	76 ± 6	71 ± 8
	2 M KCl	22 ± 5	20 ± 4
K. marxianus E1	1 M KCl	**85 ± 8**	**95 ± 2**
	1.25 M KCl	**77 ± 9**	**90 ± 1**
	1.5 M KCl	**72 ± 9**	**48 ± 7**
	2 M KCl	**15 ± 5**	**15 ± 1**
K. marxianus CBS712	1 M NaCl	83 ± 7	92 ± 2
	1.25 M NaCl	68 ± 7	76 ± 4
	1.5 M NaCl	44 ± 6	22 ± 3
	2 M NaCl	14 ± 1	10 ± 1
K. marxianus E1	1 M NaCl	**83 ± 3**	**81 ± 2**
	1.25 M NaCl	**38 ± 3**	**23 ± 3**
	1.5 M NaCl	**18 ± 4**	**13 ± 3**
	2 M NaCl	**14 ± 3**	**11 ± 1**
K. marxianus CBS712	5 mM H_2O_2	81 ± 3	72 ± 12
	7.5 mM H_2O_2	19 ± 2	10 ± 1
	10 mM H_2O_2	13 ± 1	11 ± 1
K. marxianus E1	5 mM H_2O_2	**81 ± 5**	**14 ± 3**
	7.5 mM H_2O_2	**19 ± 1**	**11 ± 1**
	10 mM H_2O_2	**14 ± 2**	**10 ± 1**
K. marxianus CBS712	3 w/v % EtOH	87 ± 4	97 ± 3
	5 w/v % EtOH	19 ± 2	22 ± 6
	6.5 w/v % EtOH	10 ± 1	10 ± 1
K. marxianus E1	3 w/v % EtOH	**41 ± 4**	**61 ± 6**
	5 w/v % EtOH	**16 ± 1**	**13 ± 3**
	6.5 w/v % EtOH	**11 ± 1**	**10 ± 1**

Table 3. Stress tolerances of the *Kluyveromyces marxianus* CBS712 parental and E1 thermotolerant mutant strain pre-cultured at 37 °C or 45 °C.

[a] - Growths recorded in stress-exposed cultures were compared to OD_{600} values read in non-stressed control cultures. All data shown here represent mean ± S.D. values calculated from three independent experiments.

Some researchers have reported that increased trehalose content inhibits certain important stress tolerant enzymes. During the investigation of *Kluyveromyces lactis*, Sampedro and Uribe (2004) found that the increased trehalose content leads to an increase in cell viscosity. This condition protects the proteins against heat stress. If the temperature decreases and this increased viscosity remains in the cells it will inhibit and damage the proteins. Consequently, if the stress is over and it is not accompanied by a quick hydrolysis of trehalose many essential enzymes can be damaged.

Therefore, persistently high trehalose level increases thermotolerance but may also sensitize yeast cells to other kinds of environmental stress.

4. New Results

- During my work I produced the thermotolerant *Kluyveromyces marxianus* E1 mutant strain, then I made the comprehensive caracterisation of the ethanol production of *K. marxianus* CBS712 parental and E1 mutant strain.

- I analized the optimal ethanol fermentation parameters of the parental and the E1 mutant strain by using Response Surface Methodology (RSM). I determined the optimal starting glucose concentration, the length of fermentation and the conversion rates which indicates the economical character of ethanol production. I observed the influence of increasing temperature and different starting glucose concentrations on ethanol production.

- The results and their comparison with literary data shows that SSF technology used for ethanol production might be more economical in the industry by using the *K. marxianus* E1 mutant strain, although to confirm these results we need further investigations.

- I carried out physiologycal studies on E1 mutant in order to find the reason of the increased thermotolerance of the strain. I investigated the amount of heat shock proteins and the reduced (GSH) and oxidized (GSSH) glutathione content of the parental and mutant strains, but I have not found any difference between the two strains in this respect. Finally I established that the increased trehalose concentration of the mutant cells is responsible for the thermotolerance of the strain. Unexpectedly, the increase of the thermotolerance was accompanied by an enhanced ethanol, osmotic and oxidative stress sensitivity.

5. The exploitability of the results

We developed the *K. marxianus* thermotolerant E1 mutant strain which is able to grow and produce ethanol at 48 ^{o}C. Although its ethanol production was lower than that of the parental strain, the increased thermotolerance should be beneficial in terms of industrial applications.

During characterisation of the fermentation parameters of the *K. marxianus* CBS712 parental and E1 mutant strain, we determined the optimal starting glucose concentration, fermentation time, ethanol production capability, conversion rate (ethanol yield) and the temperature limit of growth. The maximum ethanol concentration produced by the parental strain was 1.1 mol l^{-1} and for the mutant strain it was 1.0 mol l^{-1} (Table 1), which are comparable to the 5.5-7.2% (w/v) (1.19-1.56 mol l^{-1}) yields produced by the IMB3 strain already employed in the industry (Singh *et al.*, 1998a; Banat *et al.*, 1992).

According to the results, we developed a thermotolerant *K. marxianus* strain, which was able to produce ethanol at elevated temperatures. We found that the SSF technology, which is used for bioethanol production, would be more economical with the application of this mutant. To confirm these results we need further investigations. At first, we would like to test the bioethanol production of the E1 mutant strain in laboratory scale and, maybe, in industrial fermentation systems, in these experiments we would like to use corn starch.

During the investigation of the thermotolerance of the E1 mutant, we reached the conclusion that persistently high intracellular trehalose levels cause the increased thermotolerance of the mutant strain. In addition, we investigated the intracellular glutathione and glutathione disulphide concentration of the parental and the mutant cells, in order to find out how the heat stress influenced the cells, but we did not find any differences between the two strains in this regard. Unexpectedly, these properties were accompanied by an enhanced osmotic, oxidative and ethanol stress sensitivity.

According to these results, neither trehalose nor glutathione metabolism seem to be an expedient strategy to engineer thermotolerant *K. marxianus* strains with an increased general stress tolerance. The most reliable way to increase the thermotolerance of the *K. marxianus* E1 mutant is to control of the expression of genes which are responsible for the environmetal stress response or to increase the production of certain heat shock proteins. A further aim would be the overexpression protein of the ubiquitin under a strong promoter in the *K.*

marxianus mutant strain. Ubiquitin is a protein with small molecular weight (8.5 kDa), and plays an important role in the degradation of proteins and the regulation of cell cycle as well as in the response to environmental stress. The ethanol and osmotic stress tolerances were incerased with the overexpression of ubiquitin in *Saccharomyces cerevisiae* (Hiraishi *et al.*, 2006; Arnason és Ellison, 2004). Since in the SSF technology the yeast is exposed to extreme and varying heat, osmotic and pH conditions, an increased general stress tolerance could be a valuable feature of the mutant strain in the future.

6. References

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