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SCREENING OF DIFFERENT *KLUYVEROMYCES* STRAINS FOR SIMULTANEOUS SACCHARIFICATION AND FERMENTATION

M. BOLLÓK and K. RÉCZEY

Department of Agricultural Chemical Technology, Technical University of Budapest, H-1521 Budapest, Szt. Gellért tér 4. Hungary

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It is of big importance to find a thermotolerant yeast strain with a high tolerance against inhibitors present in steam-pretreated wood, which can be used in simultaneous saccharification and fermentation (SSF) process for bioethanol production from wood.

Five thermotolerant yeast strains belonging to the genera *Kluyveromyces marxianus* (3) and *Kluyveromyces thermotolerans* (2) were studied by growing them on agar slants and in shake flasks at different temperatures using glucose medium. The yeasts were grown in the presence of the liquid of steam-pretreated softwood as well to test the tolerance of yeasts against inhibitors formed during the pretreatment of wood. The investigated yeasts proved to be sensitive to inhibitors at high temperatures. *K. marxianus Y.00243* showed less sensitivity to inhibitors at 37 °C, *K. marxianus Y.01070* was the most thermotolerant on glucose medium, but it was too sensitive to inhibitors.

Keywords: *Kluyveromyces marxianus, Kluyveromyces thermotolerans*, steam-pretreated softwood

Use of renewable energy sources for ethanol production receives a considerable attention in researches. Bioethanol has an increasing importance as a transportation fuel and octane booster (HIMMEL et al., 1997). Among the technologies which trend to produce bioethanol from lignocellulosic materials, simultaneous saccharification and fermentation (SSF) is a well-studied, enhanced significant process. This technology is in possession of several advantages, nevertheless some drawbacks are to be solved to increase the efficiency of bioethanol production. One of the emerged problems which is associated with the SSF is the difference in optimum temperature for saccharification and fermentation which are performed simultaneously in this technology (GROHMANN, 1993). In consequence of higher temperature requirement for saccharification, there are endeavours to use thermotolerant yeasts in the SSF.

Due to economic consideration, as a significant part of total cost is the cost of raw material (VON SIVERS et al., 1994), there are increased efforts to use renewable, inexpensive lignocellulosic substrates for ethanol production. In enzymatic process, the

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pretreatment method of lignocellulosics is required to increase the accessibility of cellulose to the cellulase enzyme. Steam-pretreatment is a suitable, extensively investigated chemical method for pretreatment of lignocellulosics (CLARK & MACKIE, 1987; EKLUND et al., 1995; TENBORG et al., 1998). During pretreatment, a large amount of undesired degradation products form, which are known inhibitors of yeast cells (HATZIS et al., 1996). When lignocellulosics are used as substrates for ethanol production in SSF, the fermenting yeast must be adapted to inhibitors. *Kluyveromyces* yeasts are the most frequently investigated thermotolerant yeast strains in SSF (SZCZODRAK & TARGOŃSKI, 1989; BALLESTEROS et al., 1991; LARK et al., 1997). In the present study, five thermotolerant yeast strains belonging to the group *Kluyveromyces* were investigated for their thermotolerance on glucose medium and for their sensitivity to inhibitors present in the liquid of steam-pretreated softwood at different temperatures.

1. Materials and methods

1.1. Microorganisms

Five yeast strains, kindly obtained from the National Collection of Agricultural and Industrial Microorganisms, University of Horticulture and Food Industry (Hungary), belonging to the genera *Kluyveromyces marxianus* (*K. marxianus Y.01070*, *K. marxianus Y.00243* and *K. marxianus Y.00242*) and *Kluyveromyces thermotolerans* (*K. thermotolerans Y.00775* and *K. thermotolerans Y.00798*) were screened. Commercial baker's yeast, *Saccharomyces cerevisiae* was used as a control.

1.2. Agar slant for maintenance

The yeasts were grown on agar slant containing 1% w/v glucose, 1% w/v yeast extract (Difco), 1% w/v peptone (Difco) and 2% w/v agar (Difco). In order to investigate the sensitivity to inhibitors, the agar slant contained liquid of steam-pretreated spruce (SPS) instead of glucose in a concentration corresponding to a reducing sugar concentration of 1%.

1.3. Shake flask experiments

Three-day-old agar slants incubated at different temperatures (30, 37 and 42 °C) were used for inoculation of shake flasks (100 ml Erlenmeyer-flask, 30 ml medium). The medium employed for yeast cultivation consisted of ($g\ l^{-1}$): glucose 50, yeast extract (Merck) 2.5, peptone 5, KH_2PO_4 1, $MgSO_4$ 0.3, NH_4Cl 2 (BALLESTEROS et al., 1991). The flasks were incubated under different conditions: 300 r.p.m. 30 °C; 180 r.p.m., 35 °C; 180 r.p.m., 45 °C. The cultivations were performed for investigation

of sensitivity to inhibitors at 37 °C and 45 °C, shaking at 180 r.p.m. The medium contained 4.5 ml of liquid of SPS, which was supplemented by glucose to a total sugar content of 50 g l^{-1}.

1.4. Substrate

In some cultivations, the liquid part of steam-pretreated softwood was added to the medium in order to investigate the sensitivity of yeasts to inhibitors. The SPS was kindly supplied by the Department of Chemical Engineering I, Lund University, Sweden. The pretreatment equipment and method were described previously (PALMQVIST et al., 1996). The liquid part of the substrate was analysed by HPLC (STENBERG et al., 1998). The composition of liquid after pretreatment is shown in Table 1.

1.5. Analysis

The reducing sugar content in the shake flask cultivations was determined with the dinitrosalicilic acid method (MILLER, 1959).

The yeast cell concentration was determined by measuring the dry matter content. A five milliliter sample was taken at the end of the yeast cultivation, centrifuged at 2800 r.p.m. for 10 min, washed twice with distilled water and dried in oven at 105 °C to constant weight. After about one day, the dried sample was cooled to room temperature in a desiccator and weighed (ALBERS et al., 1996).

Table 1

The composition of the liquid of stem-pretreated spruce after pretreatment

Components	Concentration (g l^{-1})
Glucose	14.0
Mannose	18.2
HAc	5.2
HMF	2.2
Furfural	1.3

2. Results

2.1. Growth on solid and liquid glucose medium at different temperatures

Thermotolerance of yeasts was investigated by growing them on agar slant incubated at three different temperatures: 30, 37 °C and 42 °C.

Table 2

Measured reducing sugars and produced dry matters during yeast cultivations on glucose at a concentration of 50 g l^{-1} at different temperatures. Inoculations of cultivations were from agar slants incubated at 30 °C

Time (h)		0	5	10	15	17[a]/18	
Yeast	Incubation T (°C)	Glucose concentration ($g\ l^{-1}$)					Initial/Produced dry matter ($g\ l^{-1}$)
K. marxianus	30	52	42	9.6	1.0	1.2	0.07/5.6
Y.01070	35	50	30	0.9	0.9	0.9	0.07/4.8
	45	52	40	5.2	0.9	1.1	0.07/3.0
K. marxianus	30	50	39	8.8	1.2	1.2	0.21/6.0
Y.00243	35	51	28	0.8	0.8	0.8	0.21/5.1
	45	54	39	20.2	14.6	13.5	0.21/1.9
K. marxianus	30	53	39	24.9	21.2	21.8	0.32/3.9
Y.00242	35	46	25	0.9	0.9	1.0	0.32/4.1
	45	51	36	27.2	21.3	18.1	0.32/1.7
K. thermotolerans	30	53	43	14.9	1.4	1.5[a]	0.23/6.0
Y.00775	35	51	44	31.8	20.7	22.3[a]	0.23/3.4
	45	48	49	49.9	46.4	52.6[a]	0.23/0
K. thermotolerans	30	50	40	17.1	1.0	0.9[a]	0.17/4.2
Y.00798	35	50	46	37.0	31.1	30.2[a]	0.17/4.2
	45	50	50	49.4	47.5	51.0[a]	0.17/0.3
S. cerevisiae	30	44	45	23.8	8.7	3.8[a]	0.21/6.9
	35	51	44	8.2	0.8	0.8[a]	0.21/4.8
	45	50	48	48.5	46.6	48.7[a]	0.21/1.0

[a] The glucose concentration and the produced dry matter were measured after 17 h.

All five *Kluyveromyces* strains were able to grow at 30 °C on agar slant. When the agar slants were incubated at 37 °C and 42 °C, *K. marxianus Y.01070* and *K. marxianus Y.00243* were able to grow well, while *K. marxianus Y.00242* showed a weak growth. Slight growth of *K. thermotolerans* strains was observed at 37 °C and 42 °C (data are not shown).

The growth of yeasts was also investigated on glucose medium in shake flask experiment. Three-day-old agar slants incubated at 30 °C were used for inoculation. Glucose concentrations, initial and produced dry weight data are summarized in Table 2.

All three *K. marxianus* strains and the control baker's yeast consumed glucose with the highest rate at 35 °C. The time needed for the total sugar consumption was about 10 h for the three *K. marxianus* strains and about 15 h for *S. cerevisiae*. When running the cultivation at 45 °C only the *K. marxianus* strains could consume glucose. *K. marxianus Y.01070* proved to be the best strain, consuming most of the glucose in 15 h and producing the most cell mass. After already 10 h cultivation time glucose concentration decreased from the initial 52 g l^{-1} to 5.2 g l^{-1} while this value was 20.2 and 27.2 g l^{-1} for the other two *K. marxianus* strains. According to the 18 h data, *K. marxianus Y.01070* could consume all glucose in the medium (below 1.0 g l^{-1} estimated glucose) while there was still 13.5 g l^{-1} at *K. marxianus Y.00243* and 18.1 g l^{-1} at *K. marxianus Y.00242*. There was no growth at 45 °C for the two *K. thermotolerans* and at *S. cerevisiae*. None of the two *K. thermotolerans* strains proved to be really thermotolerant. They could not grow at 45 °C and the sugar consumption was lower at 35 °C than at 30 °C for both strains. Consequently, *K. thermotolerans* strains were not investigated in the further experiments. The dry matter produced in 17 as well as 18 h were the highest in shake flasks incubated at 30 °C. Figure 1 presents the produced dry matters by different yeasts at different temperatures.

2.2. Effect of incubation temperature of inoculum on yeast cultivation

The aim of these experiments was to examine the effect of incubation temperature of yeast slants on the cultivation at elevated temperatures.

Shake flasks were inoculated from agar slants incubated at different temperatures (30 °C, 37 °C and 42 °C). The effect of incubation temperature of agar slants was studied on yeast growth as well as glucose consumption in shake flask experiments at different temperatures. Shake flask cultivations were performed on glucose medium at 30 °C, 35 °C and 45 °C with *K. marxianus Y.01070* (Fig. 2), *K. marxianus Y.00243* (Fig. 3) and *S. cerevisiae* (Fig. 4).

Glucose consumption was the fastest when *K. marxianus Y.01070* was grown at 45 °C in shake flask inoculated from 30 °C agar slant, and *K. marxianus Y.00243* and *S. cerevisiae* were grown at 35 °C in shake flask inoculated from 30 °C agar slant. In these cases, the glucose was mostly consumed in 15 h. Produced dry matter was the highest in shake flask at 35 °C when the used inoculum was agar slant grown at 35 °C. The produced dry matters in 20 h, when the initial dry matters were 0.2 g l^{-1}, are shown in Table 3.

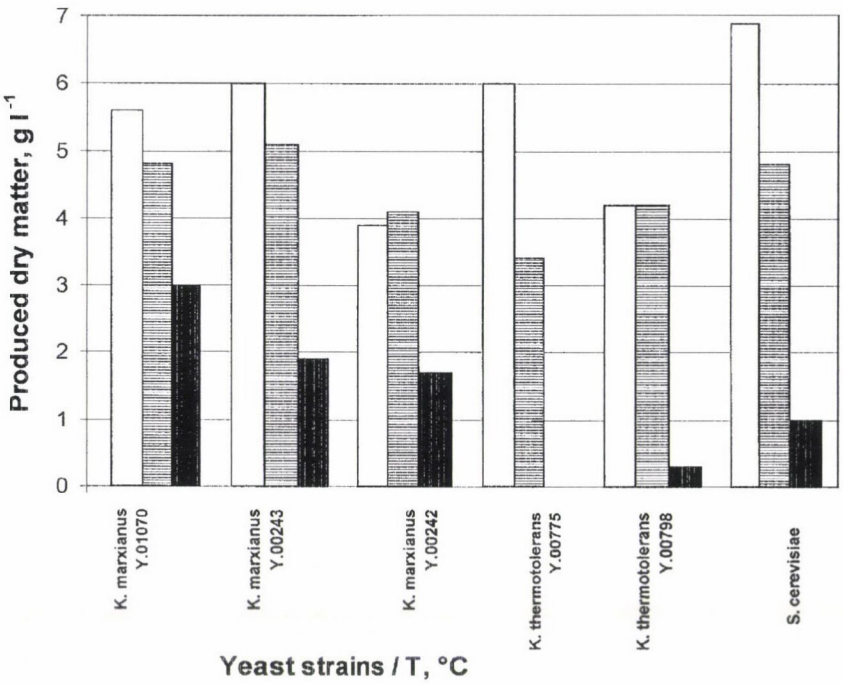

Fig. 1. Produced dry matter during yeast cultivation on glucose at a concentration of 50 g l^{-1} for different yeast strain after 18 h (*K. marxianus* strains) or 17 h of fermentation (*K. thermotolerans*, *S. cerevisiae*) at 30 °C (blank bars), 35 °C (grey bars) and 45 °C (black bars)

2.3. Growth on medium containing inhibitors

All *K. marxianus* strains were grown on agar slants containing the liquid fraction of SPS, instead of glucose, in order to investigate the tolerance to inhibitors. The agar slants were incubated at 30 °C and 37 °C. The growth on inhibitors was compared with growth of yeasts on original agar slants. The results are presented in Table 4.

None of the strains could grow at 37 °C on medium containing inhibitors. The best growth in the presence of inhibitors was observed at 30 °C in the following order: *K. marxianus Y.00243*, *S. cerevisiae*, *K. marxianus Y.01070* and *Y.00242*. All yeast strains grew more slowly on agar slant containing inhibitors than on original medium. The different yeasts were also tested in shake flask experiments at 37 °C and 45 °C on a medium containing soluble inhibitors. No growth could be observed at 45 °C. The results of cultivation at 37 °C are shown in Fig. 5.

Table 3

Produced dry matter after 20 h in shake flask experiments on glucose at a concentration of 50 g l^{-1} at different temperatures

Yeast strain	Temperature of cultivation (°C)	Incubation temperature of agar slant used for inoculation	Produced dry matter ($g\ l^{-1}$)
K. marxianus	30	30	4.4
Y.01070	35	37	5.7
	45	42	3.6
	45	30	3.3
K. marxianus	30	30	4.6
Y.00243	35	37	6
	45	42	2.4
	35	30	5.7
	45	30	2.8
S. cerevisiae	30	30	4.7
	35	37	5.4
	45	30	0

Table 4

Comparison of yeast growth on different agar slants and at different temperatures. Original agar slant contained glucose as a carbon source, "inhibitors" medium contained liquid of SPS instead of glucose in a concentration corresponding to a reducing sugar concentration of 1%

Strains	*K. marxianus*									*S. cerevisiae*		
	Y.01070			*Y.00243*			*Y.00242*					
Medium	Original		Inhibitors	Original		Inhibitors	Original		Inhibitors	Original		Inhibitors
Days	30 °C	37 °C	30 °C	30 °C	37 °C	30 °C	30 °C	37 °C	30 °C	30 °C	37 °C	30 °C
1	xx	xx	x	xx	xxx	x	xx	x	x	xxx	xx	x
2	xx	xx	x	xx	xxxx	xx	xx	x	x	xxx	xx	xx
3	xxx	xxx	x	xxx	xxxx	xxx	xxx	xx	x	xxx	xx	xx
4	xxxx	xxxx	x	xxxx	xxxx	xxx	xxxx	xxx	x	xxxx	xxx	xx
6	xxxx	xxxx	x	xxxx	xxxx	xxxx	xxxx	xxx	x	xxxx	xxx	xxx

x weak growth
xx medium growth
xxx strong growth
xxxx very strong growth

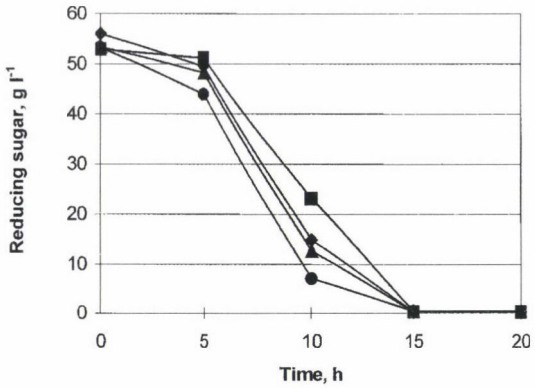

Fig. 2. Glucose consumption vs time by *K. marxianus Y.01070* on glucose medium in shake flask cultivations. When cultivations were performed at 30 °C inoculated from agar slant, maintained at 30 °C (◆); at 35 °C inoculated from agar slant, maintained at 37 °C (■); at 45 °C inoculated from agar slant, maintained at 42 °C (▲); at 45 °C inoculated from agar slant, maintained at 30 °C (●)

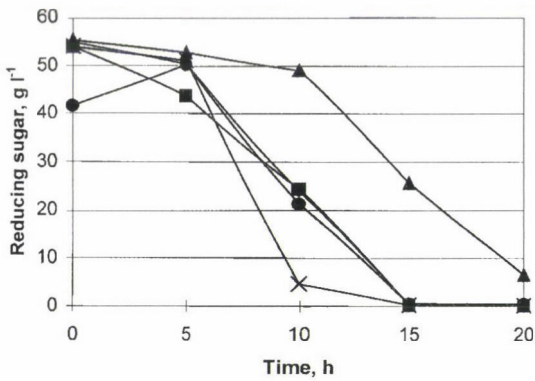

Fig. 3. Glucose consumption vs time by *K. marxianus Y.00243* on glucose medium in shake flask cultivations. When cultivations were performed at 30 °C inoculated from agar slant, maintained at 30 °C (●); at 35 °C inoculated from agar slant, maintained at 37 °C (■); at 45 °C inoculated from agar slant, maintained at 42 °C (▲); at 35 °C inoculated from agar slant, maintained at 30 °C (X)

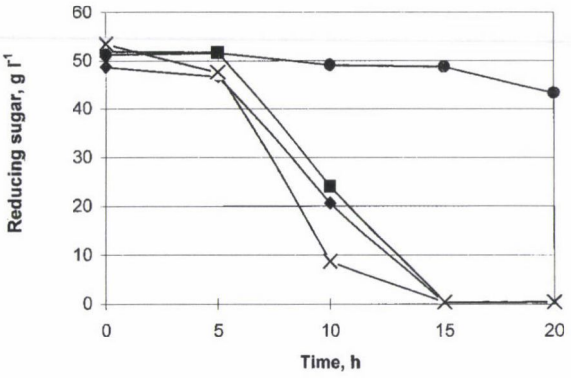

Fig. 4. Glucose consumption vs time by *S. cerevisiae* on glucose medium in shake flask cultivations. When cultivations were performed at 30 °C inoculated from agar slant, maintained at 30 °C (◆); at 35 °C inoculated from agar slant, maintained at 37 °C (■); at 35 °C inoculated from agar slant, maintained at 30 °C (X), at 45 °C inoculated from agar slant, maintained at 30 °C (●)

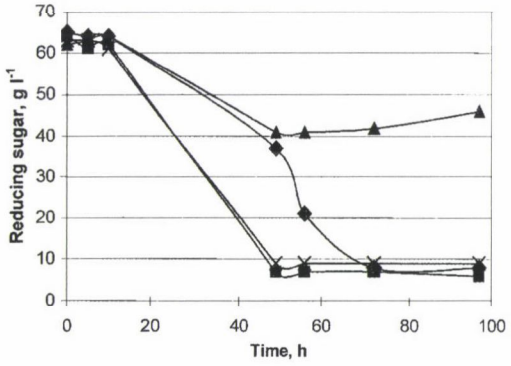

Fig. 5. Reducing sugar concentration vs time in the presence of inhibitors from SPS at 37 °C for various yeast strains: *K. marxianus Y.01070* (◆), *K. marxianus Y.00243* (■), *K. marxianus Y.00242* (▲), *S. cerevisiae* (X)

After a lag phase of about 10 h *S. cerevisiae* and *K. marxianus Y.00243* started to consume soluble sugars and reached a sugar concentration of 7–9 g l^{-1} at 50 h of fermentation. This minimum value did not change during the late phase even if the fermentation was run for 97 h. *K. marxianus Y.01070* consumed the glucose to a

concentration of 8 g l^{-1} after only 72 h of cultivation, while *K. marxianus Y.00242* was not able to use the glucose even in 97 h. The produced dry matter was the highest, 5.1 g l^{-1}, for *K. marxianus Y.00243*, when the initial dry matter was 0.23 g l^{-1}. The dry matter produced by *K. marxianus Y.01070* was 3.9 g l^{-1} with an initial dry matter of 0.31 g l^{-1}. The dry matter was 2.3 and 2.6 g l^{-1} for *K. marxianus Y.0242* and *S. cerevisiae*, when the initial dry matters were 0.16 and 0.22 g l^{-1}, respectively.

3. Conclusions

Kluyveromyces yeast strains (three *K. marxianus* and two *K. thermotolerans*) were studied, based on the examination of their growth on agar slants and in shake flask cultures at different temperatures. The sensitivity to inhibitors was also tested in some cultivations by addition of the liquid fraction of SPS to the growth medium. The two *K. thermotolerans* yeasts proved not to be really thermotolerant as they were unable to grow at 45 °C even on glucose medium. As their growth at lower temperatures was also poor, they were not studied later. On glucose medium, *K. marxianus Y.01070* showed the highest thermotolerance because it grew the fastest and produced the highest dry matter at 45 °C. At 35 °C, the produced cell mass by *K. marxianus Y.01070* and *K. marxianus Y.00243* were the same as that reached by *S. cerevisiae*. But among these strains, *K. marxianus Y.00243* gave the highest amount of cell mass at 35 °C.

Testing the effect of cultivation temperature of the used slants as inoculum, it has been proved that agar slants incubated at 30 °C are the best for all strains to inoculate in shake flask experiments. The best result was obtained with *K. marxianus Y.01070* growing at 45 °C, when the agar slant used for inoculation was maintained at 30 °C.

The presence of the liquid fraction of SPS has inhibited significantly the growth of the investigated yeasts at higher temperatures. SPS can be used as a substrate in simultaneous saccharification and fermentation (SSF) for ethanol production (STENBERG et al., 1998). In the course of the pretreatment of lignocellulosic substrate, soluble sugars such as glucose, mannose, arabinose, galactose and xylose are released from the hemicellulose fraction and undesired degradation products such as furfural, hydroxymethyl furfural, phenolic compounds, acetic acid are formed which inhibit both the growth and the fermentability of yeast cells (OLSSON & HAHN-HÄGERDAL, 1996). The above-mentioned coumpounds are present in the liquid fraction of SPS.

Using thermotolerant microorganism in SSF allows of increasing the temperature of the process which is favourable for enzymatic hydrolysis (BOYLE et al., 1997). As the degree of saccharification is the rate-limiting step in the major part of the SSF (PHILIPPIDIS & SMITH, 1995), the efficiency of ethanol production can be improved at higher temperature. In several studies (BALLESTEROS et al., 1991; BANAT et al., 1992; LARK et al., 1997) the growth and the fermenting ability of *Kluyveromyces* strains were

tested at elevated temperatures. SZCZODRAK and TARGOŃSKI (1988) have selected *Kluyveromyces* strains which were able to ferment glucose, galactose and mannose from the hydrolysis products of hemicellulose at above 40 °C. However, the sensitivity of *K. marxianus* strains to inhibitors released from the pretreatment of woody lignocellulosics has not been tested yet.

In the present study, the investigated *K. marxianus* strains could grow on agar slant containing the hydrolysate of SPS as a substrate. However, the growth was inhibited by inhibitors present in the substrate. The negative effect of inhibitors increased with increasing the temperature. Consequently, none of the yeasts was able to grow at 45 °C in the presence of inhibitors.

In shake flask experiments, all thermotolerant yeast strains were more sensitive to inhibitors at 37 °C than *S. cerevisiae* except for *K. marxianus Y.00243*. On the other hand, the produced dry matter by *K. marxianus Y.00243* was twice as high as that of *S. cerevisiae*. According to these data, *K. marxianus Y.01070* is the best in glucose utilization at 45 °C – while both this and *K. marxianus Y.00243* are good candidates at 35 °C. All three *K. marxianus* strains have consumed 98% of the added glucose at 35 °C until the 10 h fermentation while *K. thermotolerans* has had still more than half (31.8, 37 g l^{-1}) and *S. cerevisiae* 8.2 g l^{-1}. On the basis of these results, *K. marxianus Y.00243* proved to be the best thermotolerant strain which can grow at high temperature (45 °C) and has the smallest sensitivity to inhibitors at 37 °C among the investigated strains. The improvement of the inhibitor tolerance of *K. marxianus Y.00243* at higher temperatures is required in case we would like to use it effectively in the SSF experiments at elevated temperatures.

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