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ETHANOL PRODUCTION ON THE MEDIA CONTAINING GLUCOSE AND XYLOSE BY COCULTURE OF *PICHIA STIPITIS CCY* 39501 AND RESPIRATORY DEFICIENT MUTANT OF *SACCHAROMYCES CEREVISIAE* V^{30}

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ABSTRACT
INTRODUCTION
MATERIALS AND METHODS
RESULTS
DISCUSSION
CONCLUSIONS
REFERENCES

ABSTRACT

Coculture of xylose-fermenting yeast *P. stipitis* CCY 39501 and respiratory deficient mutant of *S. cerevisiae* V_{30} designated as V_{30} I 40 was used for ethanol fermentation on a medium containing glucose and xylose mixture and compared to *P. stipitis* monoculture or coculture of *P. stipitis* and *S. cerevisiae* V_{30}. Batch fermentations were carried out on a model medium or on a medium containing both sugars derived from direct saccharification

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of either wheat straw or birch sawdust. The yields obtained were 0.38 g/g, 0.34 g/g and 0.4 g/g for model medium, wheat straw and birch sawdust hydrolysates respectively, after cofermentation of *P. stipitis* with RD mutant V_{30} I 40. The results confirmed the application of this coculture for ethanol fermentation of sugars derived from lignocellulosic hydrolysates.

Key words: ethanol fermentation, xylose, lignocellulose, SSF, coculture, respiratory deficient mutant of *S. cerevisiae*, *P. stipitis*

INTRODUCTION

Plant biomass represents abundant and renewable source of carbohydrates, e.g. glucose and xylose. Ethanol obtained as a result of fermentation of these sugars is used for petrol aims as agent, which increases the octane amount of petrol or component of gasohol [21]. Efficient fermentation of sugars derived from lignocellulosic biomass by hydrolysis is one of the key processes influencing on industrial application of this process. Effective fermentation of pentoses, especially xylose by appropriate microorganisms is a problem. Among yeast strains *Pichia stipitis, Candida shehatae* and *Pachysolen tannophilus* have the ability to ferment xylose with low yields and productivities, and they are quite sensitive to ethanol concentration in the medium [20, 7, 18]. *S. cerevisiae*, a known yeast used for efficient fermentation of glucose from the ages, which shows resistance to high ethanol concentrations in the medium, is unable to ferment xylose. Coculture process, which associates fast and efficient glucose fermenting yeast (*S. cerevisiae*) with xylose fermenting yeast (*P. stipitis*) is good method to resolving this problem [12, 13]. Yeast used for xylose fermentation needs oxygen, therefore good results may be obtained by cofermentation with respiratory deficient mutant of *S. cerevisiae* which doesn't require oxygen in the medium. In such cultivation, glucose is the first sugar, which is assimilated by RD mutants of *S. cerevisiae* and after reduction of its repressing action on xylose fermentation, this pentose is assimilated by the appropriate yeast. The main reason limiting the yield during fermentation of sugar mixtures in this coculture process is the low ethanol tolerance of the xylose fermenting strains [16]. In different types of coculture process the yields obtained were in the range of 0.38 – 0.45 g of ethanol / g of consumed sugars depending on culture conditions [11, 14, 15, 16, 23]. The aim of this work was to determine whether RD mutant S. *cerevisiae* designated as V_{30}I 40 is useful for coculture with *P. stipitis* CCY 39501. Both RD mutant of *S. cerevisiae* and *P. stipitis* strain have been reported in the earlier work [9]. The applicability of these strains to ferment glucose and xylose mixture in the model medium or wheat straw and birch sawdust hydrolysates, which are the two popular waste substrates in Poland, was examined.

MATERIALS AND METHODS

Strains: *S. cerevisiae* V_{30} was obtained from the Institute of Agricultural and Food Biotechnology in Warsaw and its respiratory deficient (RD) mutant V_{30}I 40 was selected after ethidium bromide mutagenisation at the Dep. of Food Technology and Storage, Agricultural University in Lublin [10]. *P. stipitis* CCY 39501 was obtained from the Slovakia Culture Collection of Yeast. These strains were stored at 4°C on YPG or YPX agar slants, respectively.

Media

Inoculation medium used for *S. cerevisiaee* V_{30} and its RD mutant V_{30}I 40 - YPG contained (g/dm^3) yeast extract (10.0), peptone (10.0) and D-glucose (20.0), pH 5,0.

Inoculation medium for *P. stipitis*-YPX contained (g/dm^3) yeast extract (10.0), peptone (10.0) and D-xylose (20.0), pH 5,0.

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Fermentation medium (F3) (according to Laplace et al. [14] after modification) contained (g/dm^3) yeast extract (3.0), malt extract (3.0), D-xylose (15.0), D-glucose (35.0), $(NH_4)_2SO_4$ (5.0), KH_2P0_4 (3.0), pH 5,5.

Fermentation medium (F4) - like above but without sugars, it was added to lignocellulosic hydrolysates.

Fermentation of glucose and xylose on synthetic F3 medium

Inocula of yeasts were grown in test tubes with an appropriate medium at 28°C on a rotary shaker at 150 rpm for 24 h. Batch fermentations of glucose and xylose mixture were carried out in Erlenmeyer flasks containing 150 cm^3 of F3 medium and inoculated with 2% v/v of yeasts (1% v/v of either S. *cerevisiae* V_{30} (8.05±1.65 x 10^7 cells/cm^3) or RD mutant V_{30}I 40 (7.1± 0.8 x 10^7 cells/cm^3) and 1% v/v of *P. stipitis* culture (4.05±0.6 x 10^7 cells/cm^3) or 2% v/v of *P. stipitis* alone). Cultures were run at 28°C on a rotary shaker at 150 rpm for 4 days. Samples of fermentation broth were collected for analysis everyday and pH was controlled and sterilely regulated to 5.5 value using 15 % NaOH. This experiment was carried out twice.

Pretreatment of lignocellulosic materials

Wheat straw and birch sawdust were used in this experiment. Straw was cut to about 1 - 2 cm fragments and milled. 30 cm^3 of 1% HCI was added to 12 g of each biomass sample. After 18 h these samples were heat-treated in a 250 cm^3 capacity stainless steel reactor with a thermostated oil bath heater at 185°C for 8 min and 200°C for 10 min for wheat straw and birch sawdust, respectively. After hydrolysis the reactor was cooled and the biomass was steamed for 12 min (straw) or 15 min (sawdust) in order to detoxicate the samples. Each biomass was transferred to an Erlenmeyer flasks, then 50 cm^3 of sterilized F4 medium was added and the pH was adjusted to 5.0 by 30% NaOH.

Simultaneous saccharification and fermentation (SSF) of lignocellulose

2 cm^3 of cellulolytic enzymes (Celluclast, Novo Nordisk), 0.2 cm^3 of beta-glucozydase (Novozyme 188, Novo Nordisk) and yeast inoculum in a total amount of 10 % v/v (5% v/v of RD mutant V_{30}I 40 and 5% v/v of *P. stipitis)* were added on F4 medium containing plant hydrolysates. The control culture of *P. stipitis* was also prepared. Control experiment for the degree of saccharification was run without yeasts (instead of microorganisms sterile distilled water was added to the medium). Simultaneous saccharification and fermentation process was carried out on a rotary shaker at 150 rpm at 28°C for 4 days. Samples for analysis were taken before incubation and after 1, 4, 24, 48, 72 and 96 h of cultivation. The pH was controlled and corrected using 15 % NaOH. These cultures were carried out twice.

Analytical methods

Concentrations of D-glucose, D-xylose and xylitol in fermentation broth were determined by HPLC on a column Supelco (Supelconsil^{TM}) LC-NH_2 (length 25 cm) using acetonitrile: distillate water solution (80:20) as liquid phase and refractometer detection (Knauer). Ethanol was analysed according to Kellermann [8]. Yeast's biomass was monitored gravimetrically: 5 cm^3 of fermentation samples were centrifuged, washed with distilled water and dried at 104°C to a constant weight. All analyses were doubled and arithmetic averages were estimated on the measurements.

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RESULTS

Ethanol production on the synthetic medium

The results obtained are presented in Figures 1, 2 and 3 and Table 1. In the control of coculture process glucose was totally consumed after 1 day, whereas xylose utilisation by *P. stipitis* continued for 4 days. In the coculture process of *P. stipitis* with RD mutant *S. cerevisiae* V_{30} the assimilation of glucose was somewhat slower, but xylose was utilised 3 - 4 -fold faster than in the control. In the coculture fermentation of control strains, maximum concentration of ethanol was obtained after 48 h and in this case glucose was consumed completely with only 26.5% of consumed xylose. In the presence of RD mutant V_{30}, *P. stipitis* fermented xylose to ethanol because its concentration increased after complete glucose depletion. A maximum value of 18.8 g/dm^3 ethanol was assayed, which was higher by 3.55 g/dm^3, when compared with the control coculture. Xylose was used by yeast of control culture mainly for biomass production, which was higher than in coculture of *P. stipitis* and RD mutant S. *cerevisiae* V_{30} by 2 g/dm^3. Xylitol was detected in the fermentation broths only in trace amounts from 0 to 1.5 g/dm^3. But higher yields and productivities of fermentation were obtained for culture of *P. stipitis* and *S. cerevisiae* V_{30} because ethanol was produced mainly from glucose. It was reported that this hexose fermentation by *S. cerevisiae* monoculture gave higher ethanol yield when compared to glucose and xylose cofermentation by xylose fermenting yeast [4]. Also lower results of ethanol were obtained in glucose and xylose cofermentation by *P. stipitis* monoculture (Fig.1, Tab.1). This process was run for 4 days, but the concentration of ethanol obtained was lower than in coculture with RD mutant V_{30}I 40 by 5.33 g/dm^3 (28.3%), and higher amount of biomass was observed.

Fig. 1. Ethanol fermentation of glucose and xylose mixture by *P. stipitis* CCY 39501; C_x -xylose conc.; C_G-glucose conc.; C_E-ethanol conc.; C_B-biomass conc.

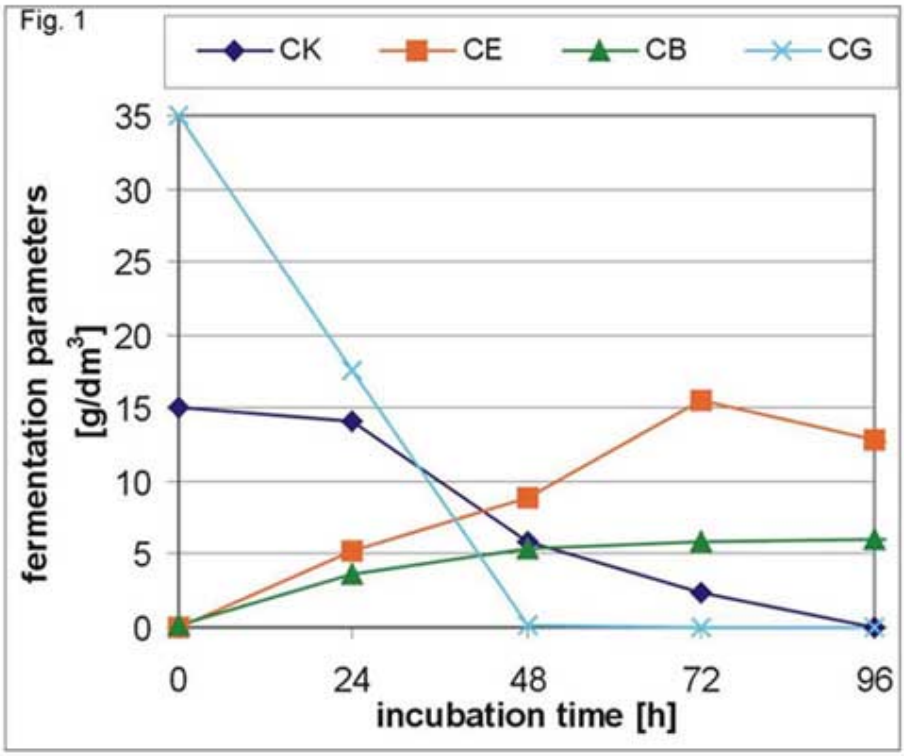

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Fig. 2. Ethanol fermentation of glucose and xylose mixture by coculture of *P. stipitis* CCY 39501 with S. *cerevisiae* V_{30}; C_x-xylose conc.; C_G-glucose conc.; C_E-ethanol conc.; C_B-biomass conc.

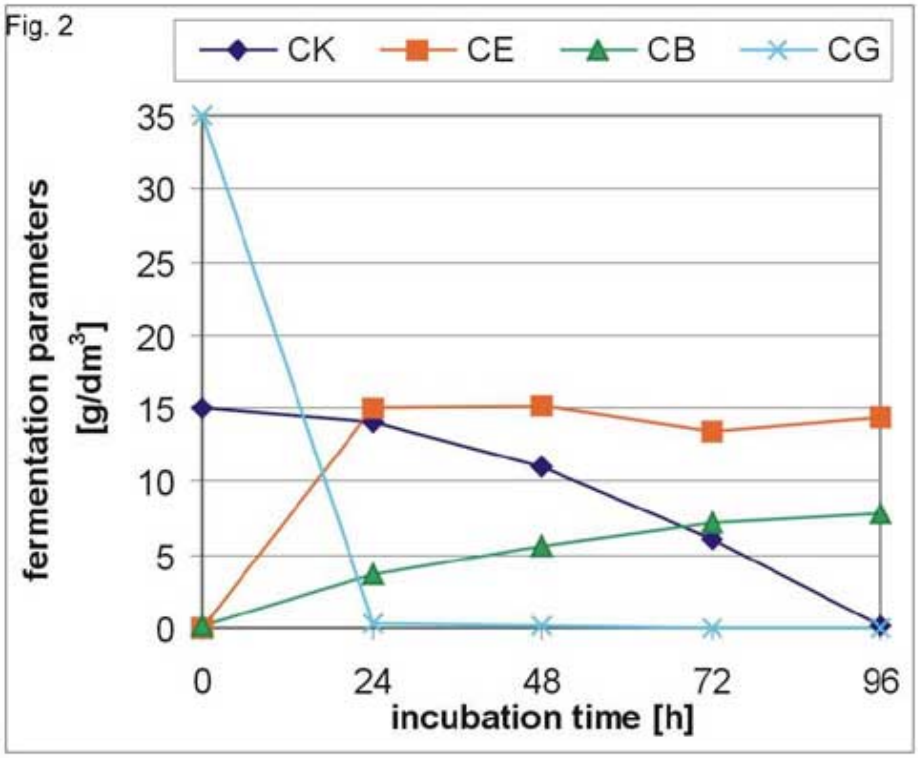

Fig. 3. Ethanol fermentation of glucose and xylose mixture by coculture of *P. stipitis* CCY 39501 with RD mutant V_{30} I 40; C_x-xylose conc.; C_G-glucose conc.; C_E-ethanol conc.; C_B-biomass conc.

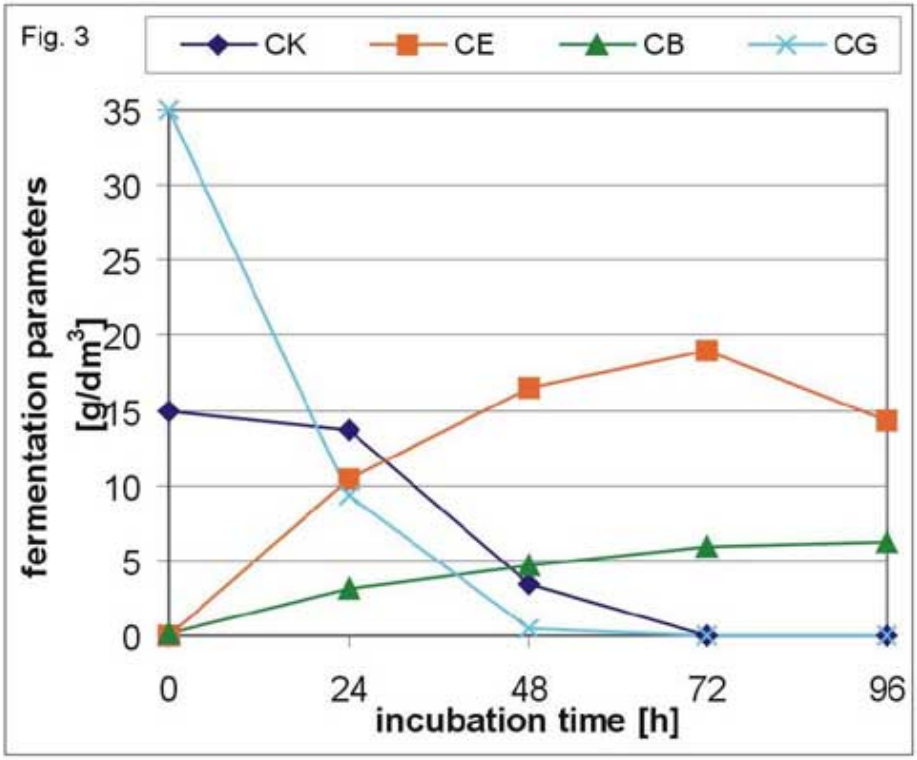

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Table 1. Parameters of glucose and xylose fermentation by *P. stipitis* CCY 39501 and coculture of *S. cerevisiae* V_{30} or RD mutant V_{30}I40 with *P. stipitis* CCY 39501

strains	S_G	S_X	max. C_E	Y_E	PY	q_E	Q_E	Y_B
P. stipitis CCY 39501	99.54	44.00	13.67	0.33	64.54	0.042	0.189	0.11
V_{30} + *P. stipitis*	99.71	26.67	15.00	0.39	76.71	0.057	0.318	0.14
V_{30}I 40 + *P. stipitis*	100.00	99.67	18.80	0.38	74.42	0.045	0.264	0.12

Nomenclature:
S_G – glucose used [%];
S_K – xylose used [%];
max. C_E – maximum ethanol concentration [g/dm^3];
Y_E – ethanol yield [g of ethanol produced / g of sugar used];
PY – practical yield [max. C_E / theoretical C_E x 100%];
q_E – specific productivity of ethanol [g of ethanol/ g of biomass x h];
Q_E – volumetric productivity of ethanol [g of ethanol/ dm^3 of medium x h];
Y_B –biomass yield [g of biomass/ g of sugar used].

Simultaneous saccharification and fermentation (SSF) of lignocellulose

Wheat straw and birch sawdust were selected on the basis of their abundance, availability and high concentration of xylose. Straw after pretreatment contained an average of 15 g/drn^3 of xylose and 4.3 g/dm^3 of glucose whereas sawdust had an average concentrations of 12.3 g/dm^3 and 8.8 g/dm^3 for xylose and glucose, respectively (Fig. 4 and 5). Control saccharification process was run with cellulases and beta-glucozydase for 4 days. During this process without yeasts 18.3 g/dm^3 of xylose and 28.0 g/dm^3 of glucose was determined in wheat straw medium (Fig. 4). The result of birch sawdust saccharification gave 14.1 g/dm^3 of xylose and 35.0 g/dm^3 of glucose (Fig. 5). During SSF process of wheat straw, the utilisation of glucose by both *P. stipitis* monoculture and coculture of this yeast with RD mutant V_{30}I 40 was fast with little or no trace amount of residual hexose in spite of continuous releasing of molecules of this sugar by enzymes (Fig. 6 and 7). After 24 h of incubation, xylose-fermenting yeast started to assimilate pentose and this continued for 3 days. The increase of ethanol concentration during the time of incubation was an evidence of xylose fermentation. In the coculture of yeasts, ethanol concentration obtained was 15.88 g/dm^3, which was higher than in *P. stipitis* culture alone by 8.1 %. The yields and productivities were also higher for coculture than for *P. stipitis* monoculture and amounted to 0.34 and 0.32 g/g, and 0.221 and 0.204 g/dm^3 x h, respectively (Table 2). It is difficult to determine what part of xylose was fermented to ethanol, because a little of it was transformed to xylitol, which was present in the fermentation broth in conc. 5 g/dm^3 and also used for new cells. Biomass concentration wasn't assayed because of heterogenic character of the medium.

Fermentation of glucose in the medium containing birch sawdust hydrolysate was slower than in the medium with wheat straw and about 2 –2.5-fold higher residual concentration of this sugar was observed in the broth. Glucose released by cellulolytic enzymes was continuously assimilated and didn't repress xylose utilization by *P.stipitis* (Fig. 8 and 9). The profile of xylose assimilation by coculture was similar to utilization of this sugar by *P. stipitis* alone. Maximum ethanol concentration in the coculture (18.69 g/dm^3) was higher than in the monoculture of *P. stipitis* only by 0.52 g/dm^3. The parameters of birch sawdust fermentation are shown in Table 3.

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Fig. 4. Saccharification of wheat straw; C_X-xylose conc.; C_G-glucose conc.

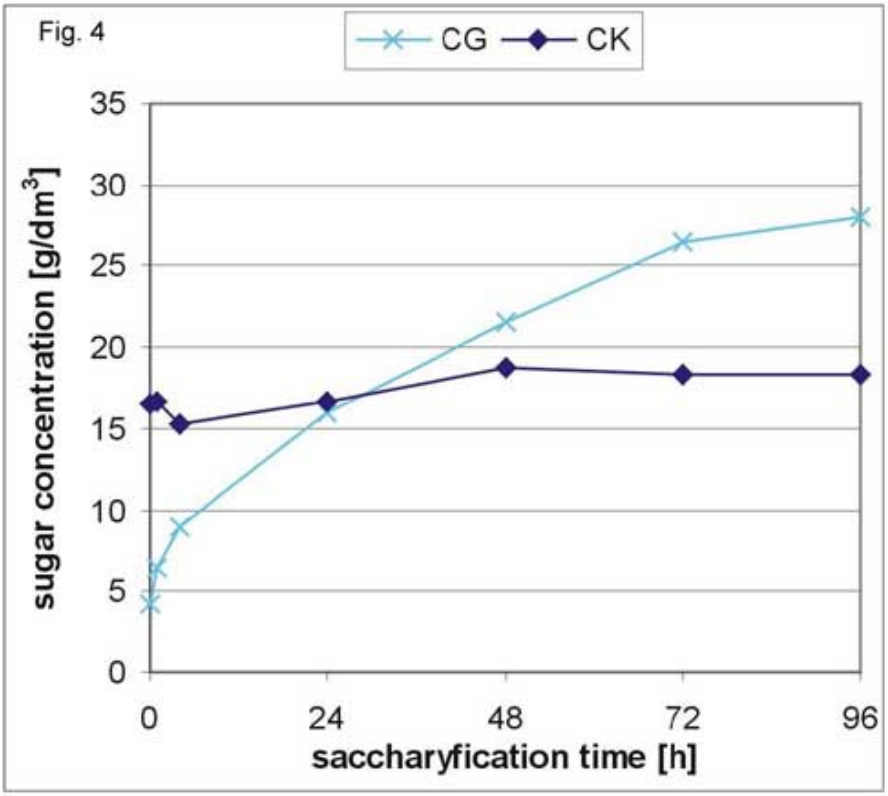

Fig. 5. Saccharification of birch sawdust; C_X-xylose conc.; C_G-glucose conc.

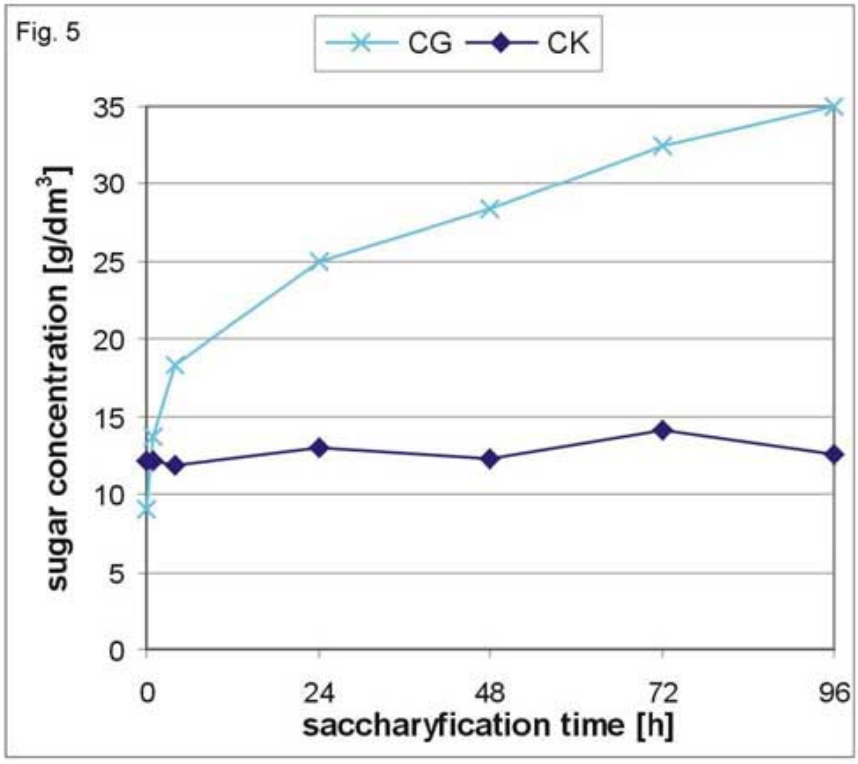

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Fig. 6. Ethanol fermentation of wheat straw hydrolysate by *P. stipitis* CCY 39501; C_x-xylose conc.; C_G-glucose conc,; C_E-ethanol conc.

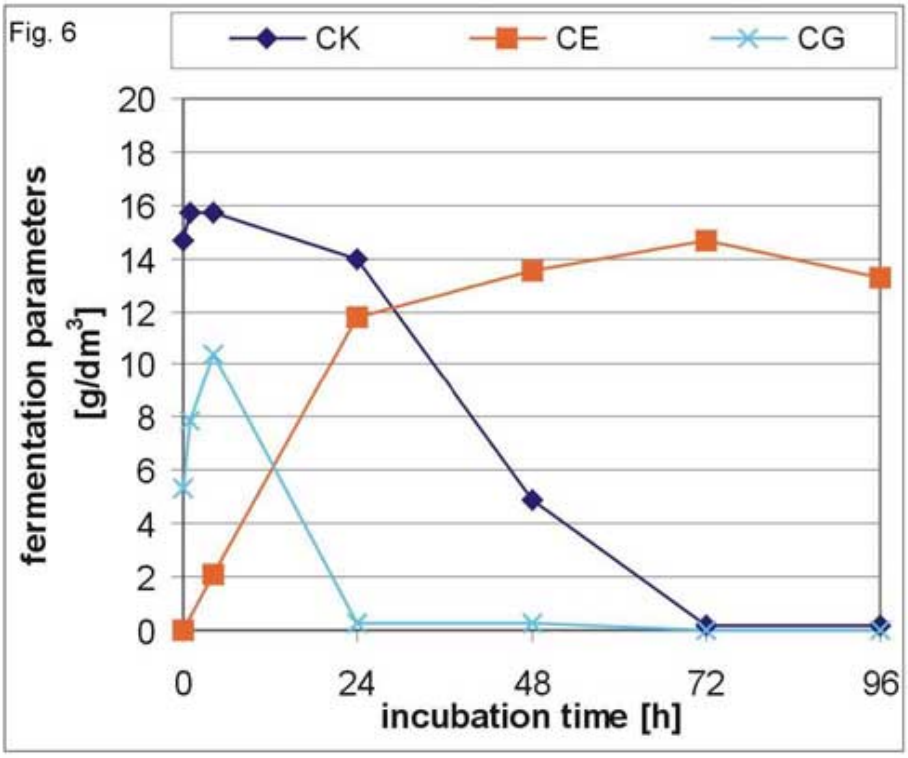

Fig. 7. Ethanol fermentation of wheat straw hydrolysate by coculture of *P. stipitis* CCY 39501 and RD mutant V_{30} I 40; C_X-xylose conc.; C_G-glucose conc.; C_E-ethanol conc.

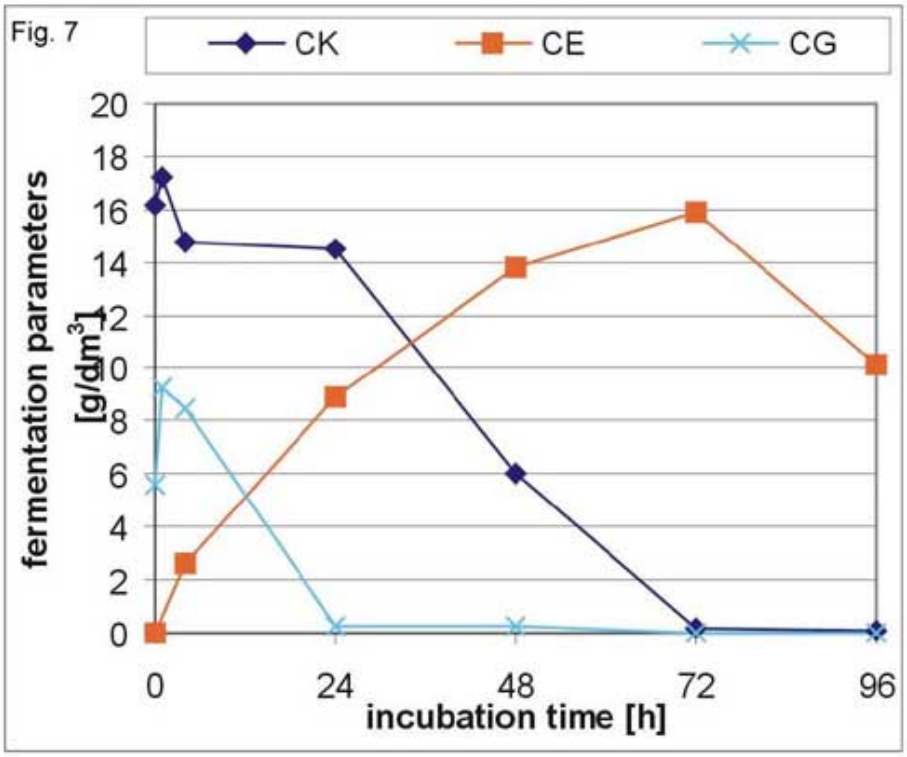

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Fig. 8. Ethanol fermentation of birch sawdust hydrolysate by *P. stipitis* CCY 39501; C_X-xylose conc.; C_G-glucose conc.; C_E-ethanol conc.

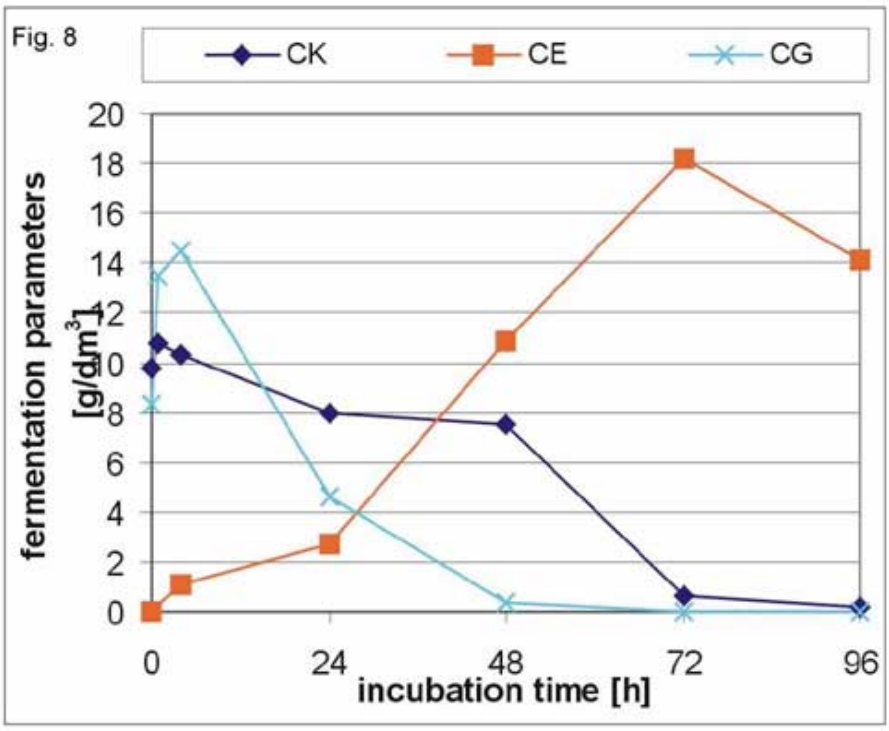

Fig. 9. Ethanol fermentation of birch sawdust hydrolysate by coculture of *P. stipitis* CCY 39501 and RD mutant V_{30} I 40; C_X-xylose conc.; C_G-glucose conc.; C_E-ethanol conc.

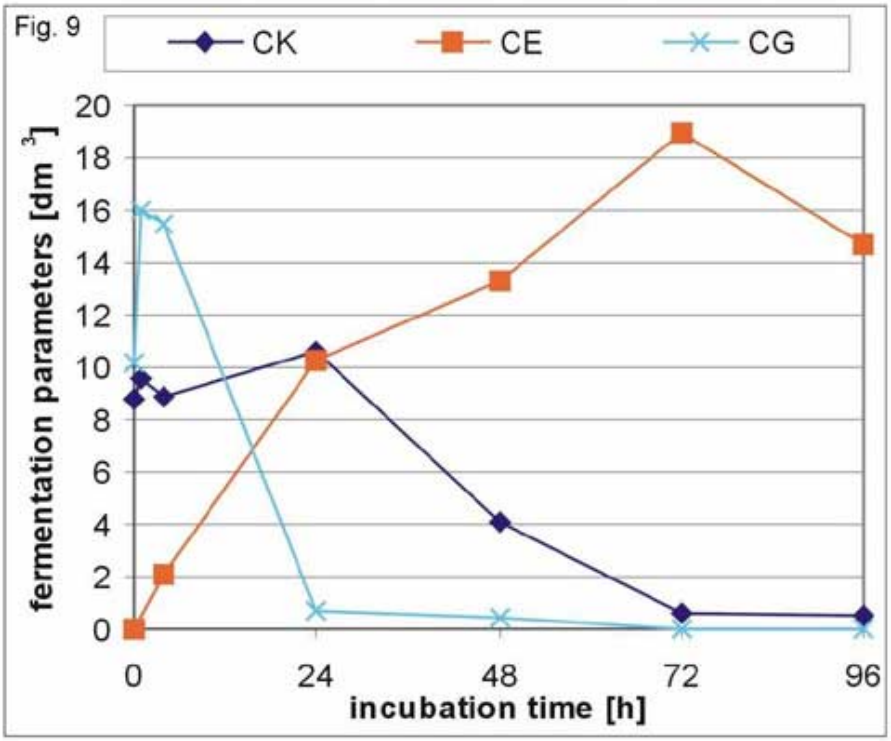

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Table 2. Parameters of wheat straw hydrolysate fermentation by selected yeasts.
Nomenclature as above

strains	S_G	S_X	max. C_E	Y_E	PY	Q_E
P. stipitis CCY 39501	100.00	98.91	14.69	0.32	62.35	0.204
V_{30}I 40 + *P. stipitis*	100.00	98.91	15.88	0.34	67.40	0.221

Table 3. Parameters of birch sawdust hydrolysate fermentation by selected yeasts.
Nomenclature as above

strains	S_G	S_K	max. C_E	Y_E	PY	Q_E
P. stipitis CCY 39501	100.00	95.24	18.17	0.39	75.64	0.252
V_{30}I 40 + *P. stipitis*	100.00	95.24	18.69	0.40	78.75	0.263

DISCUSSION

A good method, which improves yield of ethanol fermentation of glucose and xylose mixture is coculture of yeasts involving the association of a good producer of ethanol from glucose (e.g. *S. cerevisiae)* or its respiratory deficient mutant with xylose fermenting yeast (e.g. *P. stipitis*; C. *shehatae*) [4, 11, 14, 15, 16, 2, 5, 3, 23]. These yeasts may be coincubated if they don't produce killer toxins [11]. The yeasts used in this experiment were investigated for killer toxin production but no antagonistic action was observed [10]. There was no change in the concentration of ethanol during xylose fermentation by the control coculture. Similar results were obtained by Grootjen st al. [4], when a small amount of xylose was used by yeast but ethanol concentration didn't change. However, Taniguchi et al. [23] obtained only 3 g/dm^3 of ethanol during xylose fermentation by such coculture. Similar observation was made by Laplace et al. [14] during batch coculture, when *P. stipitis* produced little amount of ethanol or didn't ferment xylose at all but the biomass concentration increased. There were three reasons for this behaviour by xylose fermenting yeast: lack of oxygen was indispensable for xylose fermentation [13, 14], toxic effect generated by ethanol on *P. stipitis* due to glucose fermentation and lack of nutritive substances in the fermentation broth [19, 2]. An effective method for improving such coculture is the use of RD mutants of *S. cerevisiae*, which is disable to use dissolved oxygen in the medium. In the coculture of RD mutant V_{30}I 40 and *P. stipitis*, xylose assimilation was run for 3 days (Fig. 3) and a little amount of ethanol was produced by yeast from this pentose. An increased biomass formation was also observed in this culture, so ethanol yields were lower than yields observed for control culture. Similar ethanol concentrations (19.0 and 19.5 g/dm^3) were obtained in cultures of *P. stipitis* NRRL Y11545 with RD mutant S. *cerevisiae* CBS 1200 and *P. stipitis* NRRL 7124 with RD mutant S. *diastaticus* NCYC 625, respectively, but in this process higher yields in the range of 0.42 and 0.44 g/g were obtained, respectively [14, 15].

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Simultaneous saccharification and fermentation (SSF) of lignocellulose

This process offers some benefits compared to the conventional two-step method based on separate hydrolysis and fermentation, because one reaction medium is used, the time of this process is considerably shortened, the presence of ethanol in the fermentation broth reduces the possibility of microbial contamination and end product inhibition of cellulases is substantially diminished because glucose is continuously used by yeast. The disadvantages of such a process include different temperature and pH optima for the action of enzymes and ethanol-producing yeast, the competition between enzymes and fermenting cells and the possible inhibitory effect of ethanol on cellulases, which were shown to be significantly inhibited in medium containing above 2% w/v of ethanol [22, 17]. During the saccharification process (control), the glucose concentration increased and were 23.7 g/dm^{3}and 26.2 g/dm^3 for wheat straw and birch sawdust, respectively. Xylose concentration grew up by 2.4 and 1.8 g/dm^3, respectively. Generally, the composition of total sugars during control saccharification consisted of 60.5% glucose and 39.5% xylose for wheat straw whereas for birch sawdust had 71.3% glucose and 28.7% xylose. During saccharification of wheat straw, Zayed and Meyer [24] obtained 66.2% of glucose and 33.8% of xylose and for birch sawdust Ishihara et al. [6] obtained hydrolysates which contained glucose and xylose in average of 69.67% and 5.1%, respectively. The differences in the composition of total sugars were due to variable methods used for pretreatment and saccharification of plant biomass. Application of coculture for the fermentation of released sugars during saccharification gave better results when compared to monoculture of xylose fermenting yeast and gave higher concentration of ethanol, higher yield and productivity (Tab. 2). Coculture of *S. cerevisiae* and *P. stipitis,* as reported by Awafo et al. [1], fermented sugars derived from simultaneous hydrolysis of wheat straw with yields in the range of 0.24 –0.46 g/g, whereas ethanol yields obtained by monoculture of *P. stipitis* were in the range of 0.31- 0.49 g/g, depending on the concentration of substrate, total amount of sugar and cellulase concentration, The above mentioned author observed that RD mutant application decided about total xylose conversion in the coculture of yeasts. During continuous fermentation on non-simultaneous hydrolysis of aspen chips by coculture of *P. stipitis* NRRL Y7124 with RD mutant of *S. diastaticus,* Delgenes et al. [3] obtained an ethanol concentration of 11.5 – 13.5 g/dm^3 and yields in the range of 0.22 – 0.25 g/g with volumetric productivities of about 0.4 – 1.6 g/dm^3 x h depending on oxygen dilution rate. These observations are in good agreement with the results obtained for coculture of *P. stipitis* CCY 39501 with RD mutant V_{30} I40 cultivated on a model medium where total sugar utilization was observed in contrast to the control coculture and *P. stipitis* monoculture, with only 26.67% and 44% xylose consumption, respectively (Tab. 1). On the model medium the phenomenon of glucose catabolite repression on xylose by yeast was observed, whereas on SSF process, such repression wasn't noticed because of low concentration of glucose caused by its gradual enzymatic release. In the broth of medium containing birch sawdust, higher ethanol concentrations were obtained than in the wheat straw medium, because of higher glucose concentration after the saccharification. The results obtained in this study confirm the possibility of utilizing coculture of *P. stipitis* with RD mutant of *S. cerevisiae* for ethanol fermentation of lignocellulosic hydrolysates.

CONCLUSIONS

The fermentation of glucose and xylose mixture by the coculture of *P. stipitis* CCY 39501 with RD mutant of *S. cerevisiae* V_{30}I40 was advantageous because higher ethanol concentration by 18.9%, higher xylose assimilation by 27.76% and decrease of the fermentation time by 1 day were obtained when compared with the control coculture. Mixed culture of *P. stipitis* CCY 39501 with RD mutant signed V_{30}I40 also effectively fermented

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glucose and xylose derived from lignocellulosic hydrolysates to ethanol giving the results similar to the model medium. This suggests suitable process of the raw material pretreatment and the selection of appropriate yeasts and conditions of the ethanol fermentation.

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