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Full Length Research Paper

Mixed sugar fermentation by *Pichia stipitis*, *Sacharomyces cerevisiaea,* and an isolated xylose-fermenting *Kluyveromyces marxianus* and their cocultures

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A yeast strain with higher rates and yields in the fermentation of glucose, mannose and galactose in semiaerobic conditions than Pichia stipitis and Sacharomyces cerevisiae and ethanol tolerance than P. stipitis, was isolated from sugarcane baggase from Iranian resources. This strain that can ferment xylose with lower rates and yields than P. stipitis is characterized as Kluyveromyces marxianus. The ability of K. marxianus to ferment mixed sugars comprised of 30 g/l glucose, 30 g/l xylose, 12 g/l mannose and 8 g/l galactose (total sugar 80 g/l), as a model of many hydrolysates, were compared to P. stipitis and S. cerevisiae and then a coculture of P. stipitis and S. cerevisiae was compared with a coculture of P. stipitis and K. marxianus. In mixed sugars fermentation with individual yeasts P. stipitis shows the highest yield (0.40 gg-^{1}) and maximum ethanol (30.23 gl-^{1}), but K. marxianus shows the highest Qpmax (1.09 gl-^{1}h-^{1}) and substrate utilization efficiency (E,>99%). P. stipiti and K. marxianus coculture shows the best results with high yield (0.42 gg-^{1}) and maximum ethanol (31.87 gl-^{1}), Qpmax (1.09 gl-1h-1) and substrate utilization efficiency (E, >99%). Because of the higher rates and yields of K. marxianus to ferment hexoses than P. stipitis and its higher ethanol tolerance, K. marxianus helps the P. stipitis to reach the concentration higher than 30 g/l ethanol concentration.

Key words: Ethanol, *Pichia stipitis, Kluyveromyces marxianus,* xylose.

INTRODUCTION

Ethanol is a renewable transportation fuel, and one of the best candidates for future energy resources (Torbjon and Barbel, 1989). Moreover its use could help avoid accumulation of carbon dioxide in atmosphere and it is a suitable substitution for MTBE now used in gasoline production processes. Today, fuel ethanol in the United States is made from corn starch, but the great bulk of biomass consists of cellulose, hemicellulose, and liginin. Advanced bioethanol technology allows fuel ethanol production from the cellulose and hemicellulose, greatly expanding the renewable and sustainable resource base available for fuel ethanol production (Jeffries and Kurtsman, 1994; Du Preez, 1994).

The main fermentable sugars which release from hydrolysis of lignocellulosic biomass are glucose, xylose, mannose, galactose and arabinose, respectively (Taherzadeh et al., 1997; Jeffries and Sreenath, 1988).

There are many publications and patents about the optimization of efficiency of fermentation with the aim of industrial scale production of ethanol from lignocellulosic biomass. These efforts comprises of new native or genetically engineered microorganisms and new and improved processes. *Sacharomyces cerevisiae* is the most applied and traditional microorganism for ethanol production. It has a high ethanol tolerance, and high yields and rates of fermentation, but its inability to ferment xylose, the second most abundant sugar in nature, limits its use in biofuel production (Kotter and Ciriacy, 1993). Some yeast such as *Pichia stipitis* and *Candida shehateae* can ferment xylose and other impor-tant hexoses with relatively high yields and rates, but they have low ethanol tolerance, and ethanol concentrations above 30 to 35 g/l inhibits their reactions (Laplace, 1991).

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Table 1. The results of assimilation and fermentation tests for the isolated yeast.

Fermentation		Assimilation					
Glucose	+	Glucose	+	Mellibiose	-	D-mannitol	v
Galactose	+	Galactose	+	Raffinose	+	Salicin	+
Sucrose	+	Sucrose	+	Melizitose	-	Inositol	-
Maltose	-	Maltose	-	D-xylose	+	Citrate	-
Raffinose	+	Cellubiose	v	L-arabinose	+	Creatinine	-
Lactose	+	Trehalose	v	D-ribose	+		
Trehalose	-	Lactose	+	L- rammnose	-		

Much research has been focused on solving this hydrolysates fermentation problem. Use of sequential fermentation process, cocultures and two stage hydrolysis was examined (Jeffries, 2000). In a two stage hydrolysis process, first, hydrolysis in lower temperature and pressure, that release pentoses from hemicellulose, followed by pentoses fermentation by *P. stipitis*, then hydrolysis in higher temperature and pressures that release hexoses from cellulose followed by hexoses fermentation with *S. cerevisiae* or *Zimmomonas Mobilis* (Torget and Hsu, 1994). In coculture experiments, various combinations of yeasts were examined. Coculture of *S. cerevisiae* with *P. stipitis* has been previously studied (Grootjen et al., 1990, 1991). But coculture experiments have many limitations. For example, the aeration needs for xylose fermentation lowered the *S. cerevisiae* fermentation yield. On the other hand, existing glucose suppressed the xylose fermentation in batch cultures and when the glucose was depleted, the ethanol concentration around 30 g/l inhibits the xylose fermentation process.

In this study we use another combination of yeasts to improve the yield of fermentation. A xylose fermenting *Kluyveromyces marxianus* was isolated from the environment and its ability to ferment mixed sugar comprised of glucose, xylose, mannose and galactose was compared first with *P. stipitis* and *S. cerevisiaea* and then with cocultures of *S. cerevisiaea-P. stipitis K. marxianus-P. stipitis*. *K. marxianus* is one of the most promising yeasts in terms of biotechnological applications. Some of its strains can, and others cannot ferment xylose (Margaritis and Bajpai 1982; Stanbuk, 2003). Some of its strains have a high yield and rate of fermentation of hexoses; even higher than *S. cerevisiaa* in semiaerobic and 60 - 70 g/l total sugar concentration conditions. It has lower ethanol tolerance than *S. cerevisiae* but higher than *P. stipitis* and *C. shehateae*. Moreover it can ferment sugars in higher temperatures, around 40°C which are suitable for simultaneous sacharification and fermentation of lignocellulosic materials (Anderson et al., 1986; Barron et al., 1997; Ballesteros et al., 2004) and utilization of corn silage juice (Hang, 2003).

MATERIALS AND METHODS

Yeast strains

Commercial baker's yeast (*S. cerevisiaeae*) obtained from the France co. (*S. I. Lesaffre mareq france*), *P. stipitis CCUG18492* from Swedish collection, and the third yeast strain was isolated from sugarcane baggass (Ahvaz keshtosanaat karoon co), according to Nigam et al. (1985). Isolated yeast strain was identified according to kurtzmann et al. (1999).

Fermentation media

250 ml Erlenmayer flasks containing 100 ml culture media comprising 30 g/l glucose, 30 g/l xylose, 12 g/l mannose, 8 g/l galactose, (total sugar 80 g/l), 7 g/l yeast extract, 2 g/l ammonium sulfate, 2 g/l KH_2PO_4, 1 g /l peptone, were used in mixed sugars fermentation experiments, and pH adjusted to 4.5. Erlenmayer flasks incubated on a orbital shaker at 100 rpm for 100 h and sampling was done in 12 h intervals. For individual sugar fermentation 250 ml Erlenmayer flasks containing 100 ml culture media comprised of 20 g/l of desired sugar, 5 g/l yeast extract and 1 g/l peptone adjusted to pH = 4.5 was used.

Analytical methods

Cell density was measured turbidometrically at 600 nm. Fermentation was monitored by removing 2 ml samples. The selected samples were analyzed by high performance liquid chromatography (HPLC), equipped with UV/VIS and IR detectors (Jasco international Co., Tokyo, Japan). Ethanol was analyzed on an Aminex HPX-87H column (Bio- Rad, Richmond, CA, USA) at 60 °C with 0.6 ml/min eluent of 5 mM sulfuric acid. Glucose, mannose, xylose and galactose were analyzed on an Aminex HPX-87P column (Bio-Rad, Richmond, CA, USA) at 80^{o}C with 0.6 ml/min eluent of deionized water.

RESULTS AND DISCUSSION

Identification of isolated yeast

The isolated yeast strain was identified by morphological and physiological characteristics. Cells are ovoidal and cylindrical, psoudomycellium was formed and true hyphae were not. According to these assimilative and ferme-

Table 2. Individual sugars fermentation by three yeasts (20 g/l) is initial sugar concentration

Fermentation	Maximum ethanol (g/l)	Y (p/s)	Y (x/s)	Time of fermentation (h)
S. cerevisiaea				
Glucose	8.107	0.403	0.105	18
Xylose	0	0	0	-
Mannose	7.280	0.364	0.203	36
Galactose	6.800	0.340	0.195	48
P. stipitis				
Glucose	8.180	0.409	0.129	30
Xylose	8.141	0.404	0.147	60
Mannose	7.440	0.372	0.156	48
galactose	7.320	0.366	0.146	48
K. marxianus				
Glucose	8.429	0.421	0.140	18
Xylose	5.020	0.251	0.241	90
Mannose	8.520	0.426	0.174	24
galactose	8.240	0.412	0.126	24

ntative tests (Table 1), this isolated strain was characterized as *Kluyveromyces marxianus.* This strain produced ethanol from glucose at 40^{o}C (data were not shown).

Fermentation of individual sugars by three studied yeasts

The previous experiments show that 100 rpm is the best condition for *P. stipitis* and *K. marxianus* to ferment mixed sugars. All of the experiments were done in these conditions. Table 2 shows the results of individual sugars fermentation by three studied yeast in 100 rpm. According to Table 2 below, the following relationship exist between yeasts in yields of different sugar fermentation in 20 g/l initial sugar concentration:

Glucose: *K. marxianus* > *S. cerevisiaea* > *P. stipitis,*
Xylose: *P. stipitis* > *K. marxianus* > *S. cerevisiaea*
Mannose: *K. marxianus* > *S. cerevisiaea* > *P. stipitis*
Galactose: *K. marxianus* > *S. cerevisiaea* > *P. stipitis*

Fermentation of mixed sugars by separate cultures of *P. stipitis*, *K. marxianus* and *S. cerevisiaea*

Each fermentation media contains 80 g/l total sugars. As shown in Figure 1, *P. stipitis* shows the best fermenter in these conditions and the maximum ethanol was 30.23 g/l after 72 h. The fermentation of hexoses were slower than other yeasts; fermention of glucose and mannose were started in the first hours of fermentation and when the concentration of glucose and mannose decreased to two-thirds after 12 h, the fermentation of xylose and galactose started simultaneously. Because of the low ethanol tolerance of *P. stipitis* the reaction stopped at 30.23 g/l ethanol and about 5 g/l xylose remained intact.

K. marxianus ferments glucose and then mannose rapidly without diauxic effects; when the mannose is consumed, a short diauxic period can be distinguished and then xylose and galactose fermented completely and simultaneously. But because of its lower yield of ethanol from xylose, the maximum ethanol were obtained was 28.5 g/l after 84 h. *S. cerevisiaea* cannot ferment xylose, and when xylose comprised a main fraction of a hydrolysate, it is not a suitable candidate for fermentation of mixed sugars with high amounts of xylose.

Coculture fermentations

Figure 2 shows the fermentation of mixed sugars by coculture of *P. stipitis-S. cerevisiaea* and *P. stipitis-K. marxianus.* As seen in Figure 2 and Table 3 the coculture in the cases of *P. stipitis-K. marxianus* shows better results than *P. stipitis-S. cerevisiae*. In the case of *P stipitis-S. cerevisiae* coculture there is no improvement in maximum ethanol concentration and yield, but the fermentation time was decreased from 72 h in the case of *P. stipitis* to 60 h in coculture. Q_{pmax} decreased relative to both individual cultures of *P. stipitis* and *S. cerevisiae* and at the end of fermentation, 5% xylose was left because of the low ethanol tolerance of *P. stipitis*. It is probable that *P. stipitis* and *S. cerevisiae* have adverse effects on each other.

In the case of *P. stipitis-K. marxianus* coculture, many of the fermentation parameters show relative improvements. The maximum ethanol and yield were 30.23 g/l and 0.40 gg^{-1} in the case of *P. stipitis* and 28.5 g/l and 0.36 gg^{-1} in the case of *K. marxianus*, to 31.87 g/l and 0.42 gg^{-1} in coculture, and despite *P. stipitis*, nearly the

Table 3. Parameters for mixed sugars fermentation by *P. stipitis* and *S. cerevisiae* and *K. marxianus* and their cocultures.

Yeast(s)	Maximum ethanol (gl^{-1})	Theoritical yield	Q_{pmax} ($gl^{-1}h^{-1}$)	q_{pmax} ($gg^{-1}h^{-1}$)	μ_x	$Y_{p/s}$	$Y_{x/s}$	E, %	*Time of fermentation (h)
P. stipitis	30.23	%78	0.95	0.51	0.17	0.40	0.08	94	72
K. marxianus	28.15	%70	1.09	0.24	0.37	0.36	0.11	99	84
S. cerevisiae	14.25	%62	0.88	0.37	0.19	0.32	0.10	65	48
P. stipitis-K. marxianus	31.87	%80	1.08	0.23	0.42	0.36	0.08	99	72
P. stipitis-S. cerevisiae	29.45	%70	0.77	0.32	0.19	0.41	0.08	94	60

Q_{pmax}, maximum volumetric ethanol productivity; q_{pmax}, maximum specific ethanol productivity; μ_x, maximum specific growth rate; $Y_{p/s}$, ethanol yield; $Y_{x/s}$, cell yield, E, efficiency of substrate utilization;
*Time required for the maximum ethanol concentration to be reached.

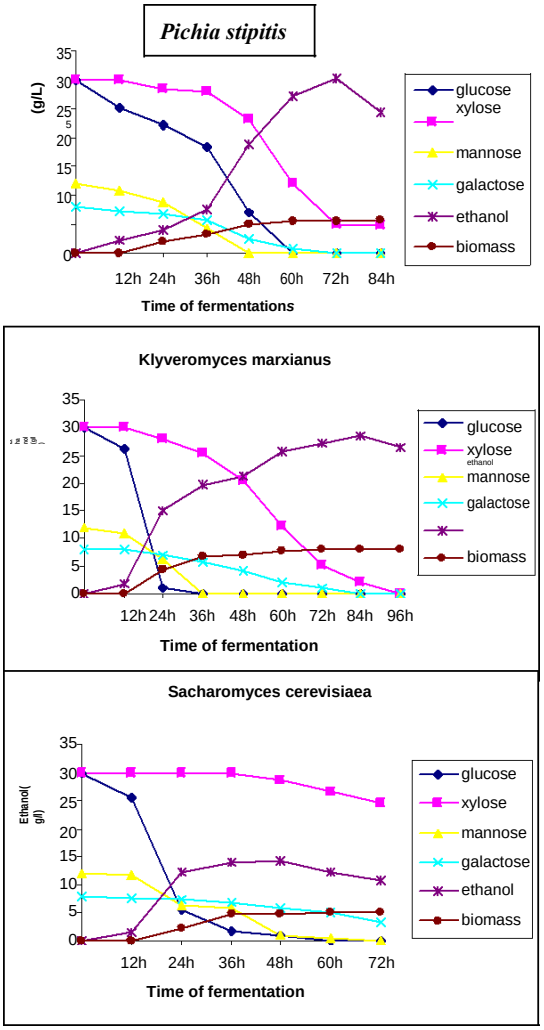

Figure 1. Fermentation of mixed sugars by individual yeasts.

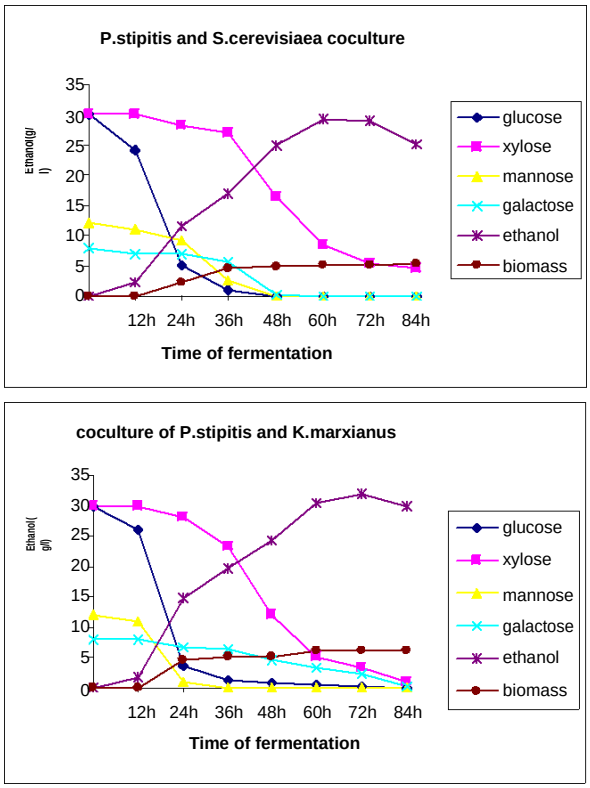

Figure 2. Fermentation of mixed sugars by cocultures of *P stipitis-S. cerevisiaea* and *P. stipitis-K. marxianus*.

sugars (80 g/l) were fermented completely (E> 99%).

P. stipitis and *K. marxianus* ferment all of the sugars that were used in this study, but *P. stipitis* ferments glucose more slowly and ferments xylose at a much higher rate than *K. marxianus*. But, *P. stipitis* were used in this study which has a lower ethanol tolerance (30 g/l ethanol) than isolated *K. marxianus* (39 g/l) (complete data were not shown). Moreover, in this study, the adverse effect of both yeasts on each other was not seen. Relative to individual culture of *P. stipitis*, in coculture experiment, *K. marxianus*, first, tend to increase in rate of hexoses ferm-

entation, and, as a result, xylose fermentation started quickly and then, when the activity of *P. stipitis* declined because of the low ethanol tolerance of around 30 g/l, *K. marxianus* continued fermentation and ferment the residual xylose, and hence, the maximum ethanol concentration and yield were improved, and efficiency of substrate utilization are from 94% to 99%. Relative to the individual culture of *K. marxianus*, in coculture, *P. stipitis* compensated for the slow rate of *K. marxianus* xylose fermentation, and the coculture gave better results than *K. marxianus* individual culture.

K. marxianus with high rates and yields of hexoses fermentation, ability to ferment xylose, ethanol tolerance more than xylose fermenting yeasts and ability to ferment sugars in high temperatures, is better than *S. cerevisiae* for coculture with *P. stipitis* and better than *P. stipitis* as the only fermenter. Evaluation of xylose fermentation ability of this strain in high temperatures and genetic modification are suitable subjects for future studies.

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