Ethanol Production by Individual and Coculture of Some Xylose and Hexoses Fermentating Yeasts

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Abstract

A xylose fermentating yeast identified as kluyveromyces marxianus isolated from sugar cane baggas tolerate ethanol and ferment glucose, mannose and galactose more than Pichia stipitis and Sacharomyces cerevisiae. The fermentation of xylose by this isolate was less than Pichia, stipitis. In mixed sugar fermentation (xylose and hexoses), Pichia stipitis with maximum ethanol (30.23 gl-1) and the yield (0.40 gg-1) was the best. However in coculture experiments, P. stipitis-K. marxianus, showed maximum substrate utilization efficiency (E≥%99) and highest Qpmax (1.09gl-1h-1). So coculcure of these two yeasts showed maximum ethanol production (31.87 gl-1).

Key word: ethanol, Pichia stipitis, Kluyveromyces marxianus,xylose.

تولید ا تانل توسط کشت منفرد و توام مخمرهاي تخمیر کننده زایلوز و هگزوزها

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مخمر کلیورومایسس مارکسیانوس تخمیر کننده زایلوز ازباگاس جداسازي و شناسایي گردید. این مخمر تحمل اتانل و تخمیر گلوکز، مانوز و گالاکتوز بیشتري نسبت به پیچیا استي پیتیس و ساکارومایسس سرویزیه دارد. تخمیر زایلوز توسط این سویه کمتر از پیچاستي پیتیس بود. درتخمیر قند مخلوط از (زایلوزوهگروزها) پیچیااستي پیتیس با ماکزیمم تولید اتانل $۳۰/۲۳ gl^{-1}$ و راندمان $۰/۴۰ gg^{-1} h^{-1}$ بهترین سویه شناخته شد. در

كشت توام پيچيا استي پيتيس و كليورومايسس ماركسيا نوس كارائي مصرف سوبسترا ۹۹% و ماكزيمم بهره‌وري مخصوص اتانول $g1^{-1}h^{-1}$ ۱/۰۹بدست آمد. كشت توام اين دو مخمر بيشترين توليد اتانل gl^{-1} ۳۱/۸۷ را نشان داد.

واژه‌هاي كليدي: اتانل، پيچيااستي پيتيس، كليورومايسس ماركسيانوس، زايلوز

1. Introduction

Ethanol is a renewable transportation fuel, and one of the best candidates for future energy resources (1), moreover, its use could help avoid accumulation of carbon dioxide in atmosphere and it is a suitable substitutions of 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 (2,3).

Main fermentable sugars released from hydrolysis of lignocellulosic biomass are glucose, xylose, mannose, galactose and arabinose, respectively (4, 5).

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 comprise 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, as well as 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(6). Some yeasts such as *Pichia stipitis* and *Candida shehateae* can ferment xylose and other important hexoses with relatively high yields and rates, but they have low ethanol tolerance, and ethanol concentrations above 30 to 35 gl^{-1} stopp their reactions (7).

Many studies have focused on solving this hydrolysates fermentation problem. Use of sequential fermentation process, cocultures and two stage hydrolysis were examined (8). In the two stage hydrolysis process, first, hydrolysis in lower temperature and pressure released pentose from hemicellulose, followed by pentose fermentation by *Pichia stipitis*, then hydrolysis in higher temperature and pressures that release hexoses from cellulose followed by hexoses fermentation with *S. cerevisiae* or *Zimmomonas mobilis*(9). In coculture experiments, some combination of yeasts was examined. Coculture of *S.cerevisiae* with *P.stipitis* have been studied previously (10,11), but coculture experiments have many limitations. For example, aeration is needed for xylose fermentation and this condition reduces the *S.cerevisiaea* fermentation yield.

Table 1- The results of assimilation and fermentation tests for 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	-		

On the other hand, existed glucose suppressed the xylose fermentation in batch cultures and when the glucose depleted, the ethanol concentration around 30 gl^{-1} decreased or declined the xylose fermentation process.

In this study, we used another combination of yeasts to improve the yield of fermentation.

A xylose fermenting *K.marxianus* was isolated from environment and its ability to ferment mixed sugar comprising glucose, xylose, mannose and galactose compared with *P.stipitis* and *S.cerevisiaea* and then cocultures of *S.cerevisiaea-P.stipitis* and *K.marxianus-P.stipitis* were examined in batch cultures to reach the better combination.

Kluyveromyces marxianus is one of the most promising yeasts with biotechnological applications. Some of its strains can ferment xylose, but some other can not (12,13). Some of its strains have a high yield and rate of fermentation of hexoses even higher than *S.cerevisiaa* in semi aerobic and 60-70 gl^{-1} 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 ºC, suitable for SSF processes (14, 15). It is used for simultaneous sacharification and fermentation of lignocellulosic materials (18), and utilization of corn silage juice (19).

2-Materials and methods

Yeast strains: Commercial baker's yeast *(Sacharomyces cerevisiaeae)* was obtained from France co.(*S.I.Lesaffre mareq france*), *Pichia stipitis CCUG18492* from Swidish collection, and the third yeast strain was isolated from sugarcane baggass (Ahvaz Keshtosanaat Karoon co.), according to Nigam J. N. *et al*.(16). Isolated yeast strain was identified according to Kurtzmann *et al* (17).

Fermentation media: 250 ml Erlenmeyer flasks containing 100 ml culture media comprising 30gl^{-1} glucose, 30gl^{-1} xylose, 12gl^{-1} mannose, 8gl^{-1} galactose, (total sugar 80gl^{-1}), 7 gl^{-1}yeast extract, 2 gl^{-1}ammonium sulfate, 2 $gl^{-1}KH_2PO_4$, 1gl^{-1} peptone were used in mixed sugars fermentation experiments, and pH was adjusted to 4.5 . Erlenmeyer flasks incubated on a orbital shaker at 100 rpm for 100 hours and sampling was done in 12 hour intervals. For individual sugars fermentation 250 ml Erlenmeyer flasks containing 100 ml culture media comprising 20gl^{-1} of desired sugar, 5 gl^{-1}yeast extract and 1gl^{-1} peptone adjusted to pH=4.5 and incubated as discussed later.

Analytical methods: Cell density was measured turbidometrically at 600 nm. Fermentation was monitored by removing 2ml 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 ºC with 0.6 $ml.min^{-1}$ eluent of 5mM sulfuric acid. Glucose, mannose, xylose and galactose were analyzed on an Aminex HPX-87P column (Bio-Rad, Richmond, CA,USA) at 80 ºC with 0.6 $ml.min^{-1}$ eluent of deionized water.

3-Results and Discussion:

Identification of isolated yeast: The isolated yeast strain was identified by morphologic and physiologic characteristic. Cells are ovoidal and cylindrical, psoudomycellium was formed and true hyphae was not. According to these and assimilative and fermentative tests (table 1), this isolated strain characterized as *Kluyveromyces marxianus.*This strain produced ethanol from glucose in 40 ºC (data were not shown).

2-Fermentation of individual sugars by three studied yeast: The previous experiments have shown that 100rpm is the best condition for *p.stipitis* and *K.marxianus* to ferment mixed sugars. All of experiments were done in these conditions. Table 2 shows the results of individual sugars fermentation by three studied yeast in 100rpm.

According to table 2 relations between yeasts in yields of different sugar fermentation in 20 gl^{-1} initial sugar concentration are as follow:

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*

3-Fermentation of mixed sugars by separate cultures of *P.stipitis, K.marxianus* and *S.cerevisiaea*: Each fermentation media contains 80 gl^{-1} total sugars. As shown in figure 1, *P.stipitis* shows the best fermenter in these conditions and the maximum ethanol was 30.23 gl^{-1} after 72h. The fermentation of hexoses were slower than other yeasts, fermentation of glucose and mannose started in the first hours of fermentation and when the concentration of glucose and mannose decreased to two third after 12 hour, the fermentation of xylose and galactose started simultaneously. Because of the low ethanol tolerance of *P.stipitis* the reaction stopped in 30.23 gl^{-1} ethanol and about 5 gl^{-1} xylose were remain intact.

Table2- Individual sugars fermentation by three yeasts. The Initial sugar concentration is 20 gl^{-1}

	Maximum ethanol (gl^{-1})	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

Time required for the maximum ethanol concentration to be reached. $Y_{p/s}$, Ethanol yield; $Y_{x/s}$, cell yield.

K.marxianus ferment glucose and then mannose rapidly without diauxic effects, when the mannose 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 28.5 gl^{-1} after 84h. *S.cerevisiaea* can not 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.

4-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, coculture in the case of *P.stipitis-K.marxianus* shows slightly 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 from 72 h, in the case of *P.stipitis* decreased to 60 hours in coculture. Of course, Q_{pmax} were decreased relative to both individual cultures of *P.stipitis* and *S.cerevisiae* in the end of fermentation, %5 xylose was left because of the low ethanol tolerance of *P.stipitis*, it was probable that *P.stipitis* and *S.cerevisiae* shown adverse effects on each other.

In the case of *P.stipitis-K.marxianus* coculture, many of fermentation parameters show relative improvements. The maximum ethanol and yield were exceed from 30.23 gl^{-1} and 0.40 gg^{-1} in the case of *P.stipitis* and 28.5 gl^{-1} and 0.36 gg^{-1} in the case of *K.*marxianus , to 31.87 gl^{-1} and 0.42 gg^{-1} in coculture, and despite *P.stipitis*, nearly all the sugars (80 gl^{-1}) were fermented completely (E≥%99).

P.stipitis and *K.marxianus* ferment all of the sugars were used in this study, but *P.stipitis* ferment glucose slower and ferment xylose with very higher rates than *K.marxianus*. But, *P.stipitis* was used in this study has a lower ethanol tolerance (30 gl^{-1} ethanol) than isolated *K.marxianus* (39 gl^{-1})(complete data were not shown), moreover in this study adverse effect of both yeast against each other were not seen. Relative to individual culture of *P.stipitis*, in coculture experiment, *K.marxianus*, first, tend to increase in rate of hexoses fermentation, and, as a result, xylose fermentation started quickly and then, when the activity of *P.stipitis* declined because of the low ethanol tolerance around 30 gl^{-1}, *K.marxianus* continue fermentation and ferment the residual xylose and hence, the maximum ethanol concentration and yield were improved, and efficiency of substrate utilization are exceed from %94 to %99. Relative to individual culture of *K.marxianus*, in coculture, *P.stipitis* compensate the slow rate of *K.marxianus* xylose fermentation, and coculture reach

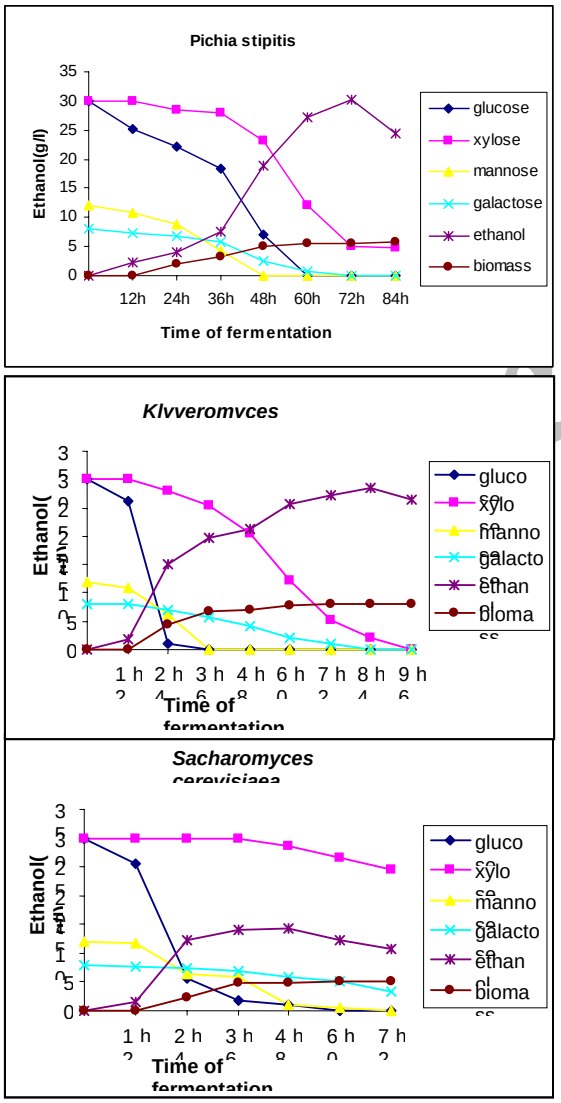

Figure1: Fermentation of mixed sugars by individual yeasts

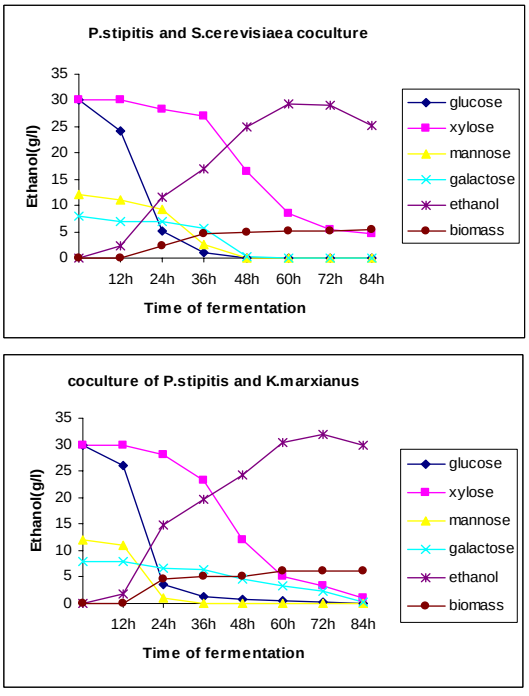

Figure 2: Cocultures between *P.stipitis-S.cerevisiaea* and *P.stipitis-K.marxianus*.

Table3- Parameters for mixed sugars fermentation by *P.stipitis* and *S.cerevisiae* and *K.marxianus* and their cocultures

	Max ethanol(gl^{-1})	Theoretical 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*

to better results than *K.marxianus* culture individually. *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 of it, is the suitable candidates for future studies.

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