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ORIGINAL PAPER

Ethanol and xylitol production from glucose and xylose at high temperature by *Kluyveromyces* sp. IIPE453

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Abstract A yeast strain *Kluyveromyces* sp. IIPE453 (MTCC 5314), isolated from soil samples collected from dumping sites of crushed sugarcane bagasse in Sugar Mill, showed growth and fermentation efficiency at high temperatures ranging from 45°C to 50°C. The yeast strain was able to use a wide range of substrates, such as glucose, xylose, mannose, galactose, arabinose, sucrose, and cellobiose, either for growth or fermentation to ethanol. The strain also showed xylitol production from xylose. In batch fermentation, the strain showed maximum ethanol concentration of 82 ± 0.5 g l^{-1} (10.4% v/v) on initial glucose concentration of 200 g l^{-1}, and ethanol concentration of 1.75 ± 0.05 g l^{-1} as well as xylitol concentration of 11.5 ± 0.4 g l^{-1} on initial xylose concentration of 20 g l^{-1} at 50°C. The strain was capable of simultaneously using glucose and xylose in a mixture of glucose concentration of 75 g l^{-1} and xylose concentration of 25 g l^{-1}, achieving maximum ethanol concentration of 38 ± 0.5 g l^{-1} and xylitol concentration of 14.5 ± 0.2 g l^{-1} in batch fermentation. High stability of the strain was observed in a continuous fermentation by feeding the mixture of glucose concentration of 75 g l^{-1} and xylose concentration of 25 g l^{-1} by recycling the cells, achieving maximum ethanol concentration of 30.8 ± 6.2 g l^{-1} and xylitol concentration of 7.35 ± 3.3 g l^{-1} with ethanol productivity of 3.1 ± 0.6 g l^{-1} h^{-1} and xylitol productivity of 0.75 ± 0.35 g l^{-1} h^{-1}, respectively.

Keywords *Kluyveromyces* sp. · Ethanol · Xylitol · High temperature · High cell stability

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Introduction

Rising prices of crude oil coupled with increasing demand for transportation fuel is the major constraint in the economic development of many nations. Scarcity of fossil fuel has led to the use of ethanol-blended gasoline (20–80%) in Brazil and the USA. Ethanol acts as an octane booster and minimizes carbon (CO) and nitrous oxide (NO_x) emission from tail pipes of cars [1, 15]. In this context, lignocellulosic biomass is favorable feedstock for ethanol production based on life-cycle analysis of the carbon-neutral process [15]. However, fermentation of different sugars—such as glucose, xylose, mannose, galactose, arabinose, cellobiose, and so on, that are produced by saccharification of lignocellulosic biomass—to ethanol has limitations to well-known ethanologens such as *Saccharomyces cerevisiae* or *Zymomonas mobilis* due to their metabolic inefficiency [7, 22]. The use of both cellulose and hemicellulosic sugars—such as hexose, pentose, and others that are present in a typical lignocellulosic biomass hydrolysate—is essential for the economical production of ethanol [19, 24, 25]. Therefore, microorganisms that are able to ferment both glucose and xylose are required for efficient bioconversion of biomass to ethanol [19, 22].

Metabolic engineering of known ethanologens by introducing essential genes expressing xylose reductase (*XR*), xylose dehydrogenase (*XD*), and xylulokinase (*XK*) from *Pichia stipitis* in *Saccharomyces cerevisiae* [12, 22]; xylose isomerase (*xylA*), xylulose kinase (*xylB*), transketolase (*tktA*), and transaldolase (*talB*) from *Escherichia coli* in *Zymomonas mobilis* [7, 34]; and pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adh*) from *Zymomonas mobilis* in *Escherichia coli* [7, 15] has been reported. Yet further improvements on stability of such genetically modified microorganisms are required for large-scale commercial production of bioethanol. Most of the potential ethanologens in industrial use belong to the mesophilic group (28–35°C), whereas thermophilic ethanologens have certain advantages over mesophiles. Solvent tolerance, energy saving through reduced cooling cost, higher saccharification and fermentation rate, easier stripping of ethanol from broth, and minimum risk of contamination are the major advantages of thermophilic ethanologens [4, 21].

Various thermophilic bacteria such as *Thermoanaerobacter ethanolicus* [11], *Thermoanaerobacterium saccharolyticum* [27], *Clostridium thermocellum* [20], and thermotolerant yeast *Kluyveromyces* sp. [3–6, 9, 28] have been used to ferment hexose and pentose sugars to ethanol. It is difficult to maintain strict anaerobic conditions in large-scale fermentations restricting the use of thermophilic anaerobes, whereas the facultative aerobes such as *Kluyveromyces* sp. have the potential for industrial applications.

Xylitol, a sugar alcohol of the polyol family, is a naturally occurring 5-carbon polyol sweetener found in fruits and vegetables and produced in the human body during normal metabolism [31]. It has the same sweetness and one third the caloric content (i.e. 2.4 Kcal g^{-1}) of sucrose [10, 17]. It has unique pharmacological properties such as prevention of tooth decay and ear infection in children; it is used as a sugar substitute for diabetic patients and in parenteral application to trauma patients [14, 29]. A number of yeasts and molds can produce xylitol from xylose because they possess the enzyme xylose reductase. *Candida guilliermondii* [10], *Candida tropicalis* [14, 16], *Candida boidinii* [31], *Candida magnoliae* [29], *Debaryomyces hansenii* [26], and *Pichia stipitis* [13] are some of the yeasts with xylitol production capability.

In this paper, we report isolation of the thermotolerant yeast, *Kluyveromyces* sp. IIPE453 (MTCC 5314), which ferments glucose, mannose, galactose, xylose, arabinose, sucrose, cellobiose, and lactose to ethanol at 50°C. Further, the strain could simultaneously ferment glucose and xylose in a mixture to ethanol and xylitol at 50°C in a batch and continuous process by recycling the cells.

Materials and methods

Microorganisms and culture conditions

Thermophiles were isolated from soil samples collected from dumping sites of crushed sugarcane bagasse in Sugar Mill. One yeast strain was selected because of its higher sugar consumption rate and high ethanol production rate. The yeast strain was identified as *Kluyveromyces* sp. IIPE453, ascomycetous yeast of the fungal family Saccharomycetaceae, order Endomycetales, by using Biolog. For growing the isolated strain *Kluyveromyces* sp. IIPE453, salt medium (SM) was used in g l^{-1}, di-sodium hydrogen ortho phosphate, 0.15; potassium di-hydrogen ortho phosphate, 0.15; ammonium sulphate, 2.0; yeast extract, 1.0; carbon source, e.g. glucose, xylose, 10. The temperature and pH were optimized for growth. The optimum temperature and pH were 50°C and 5.0, respectively. The cells were grown in 250-ml flasks in a shaker at 50°C and 150 rpm on glucose, mannose, galactose, xylose, arabinose, sucrose, cellobiose, and lactose 10 g l^{-1} each separately for 24 h. The cells were also produced in large quantity by growing in Bioflow-110 bioreactor (ca. 2 l) on glucose and xylose. The dissolved oxygen was optimized without control, at 20%, 40%, and 60% of saturation. The maximum growth rate was obtained at 40% and 60% of saturation. The temperature, pH, and dissolved oxygen were controlled at 50°C, 5.0, and 40% of saturation, respectively, during the process.

Fermentation conditions

The medium for fermentation was the same as that for the growth medium, except for ammonium sulphate 1.0 g l^{-1} and carbon source, e.g., glucose, xylose as per the experiment. Temperature and pH were optimized for ethanol fermentation and were found to be the same as in growth. Fermentation was carried out in 250-ml capped flasks at 100 rpm on glucose, mannose, galactose, xylose, arabinose, sucrose, cellobiose, and lactose 20 g l^{-1} each separately for 18 h using previously grown cells of *Kluyveromyces* sp. IIPE453. Batch fermentations were carried out in Bioflow-110 bioreactor (ca. 2 l) by *Kluyveromyces* sp. IIPE453. The temperature and pH were controlled at 50°C and 5.0, respectively, during the process. Fermentations were performed with different glucose concentrations, such as 50, 100, 200 g l^{-1}, different xylose concentrations, such as 20, 30, 40 g l^{-1}, and a mixture of glucose (75 g l^{-1}) and xylose (25 g l^{-1}). Continuous fermentation was carried out in a Bioflow-110 bioreactor (ca. 2 l) by recycling the cells, with continuous feeding of glucose (75 g l^{-1}) and xylose (25 g l^{-1}) mixture at a dilution rate 0.1 h^{-1}. Feeding of the sugar solution was started after 18 h. The 60% of cells collected by gravitational settling were recycled back into the

bioreactor. The temperature and pH were controlled at 50°C and 5.0, respectively, during the process.

Analytical methods

Sugars and xylitol were analyzed by high-performance liquid chromatography (HPLC) using High Performance Carbohydrate Column (Waters) at 30°C with acetonitrile and water mixture (75:25) as the mobile carrier at a flow rate 1.4 ml min^{-1} and detected by a Waters 2414 refractive index detector. Ethanol was analyzed by gas chromatography using Ashco Neon II Gas Analyzer with a 2-m-long and 1/8-in. diameter Porapak-QS column with mesh range 80/100. The sample was injected at an inlet temperature 220°C, oven temperature 150°C, and flame ionization detector temperature 250°C using nitrogen gas as a carrier. Furfural and hydroxymethyl furfural were measured by Double Beam UV–VIS Spectrophotometer 2600 at 277 nm.

Results

Among the various thermophilic and thermotolerant microorganisms, one yeast strain, identified as yeast *Kluyveromyces* sp. IIPE453 (deposited in the Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh (India) with deposit no. MTCC 5314) showed growth and fermentation on glucose, mannose, galactose, xylose, sucrose, cellobiose, and lactose (Table 1). The optimum temperature and pH for growth and fermentation were 50°C and 5.0, respectively (Fig. 1). The strain showed maximum cell-mass yield of 43 ± 0.05% on xylose and maximum ethanol yield of 50.5 ± 0.5% on sucrose. The arabinose was used only for growth by the strain.

In the growth study, the yeast *Kluyveromyces* sp. IIPE453 produced a cell-mass concentration of 3.5 ± 0.2 g l^{-1} besides and ethanol concentration of 4.9 ± 0.3 g l^{-1} on glucose, whereas the strain could not produce ethanol as well as xylitol on xylose aerobically, as shown in Fig. 2a, b. The maximum cell-mass concentration on xylose was 7.9 ± 0.3 g l^{-1} in 24 h. The cell-mass yield $Y_{X/S}$ on glucose and xylose was obtained with 0.2 ± 0.1 g cells g^{-1} glucose and 0.43 ± 0.1 g cells g^{-1} xylose, respectively, at 50°C.

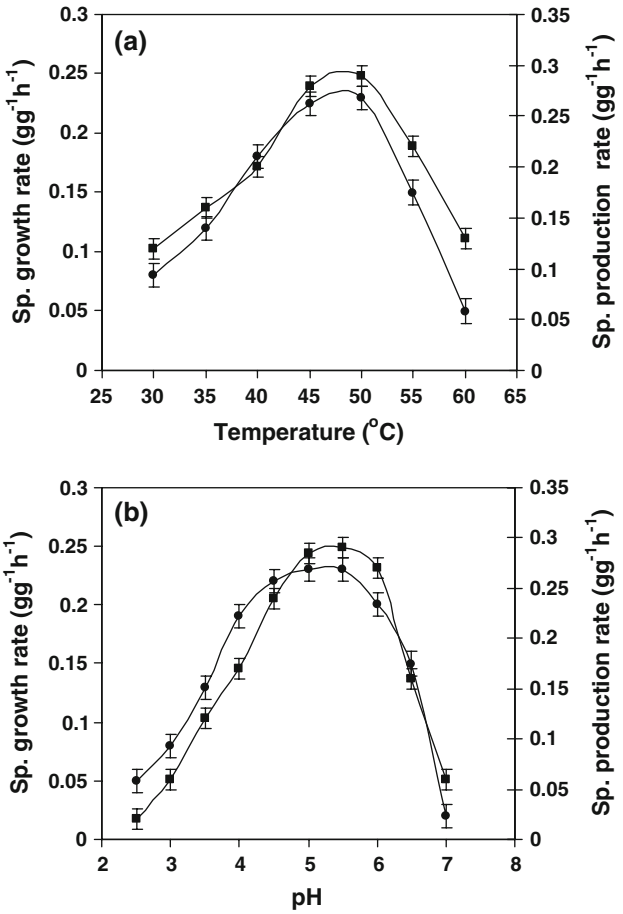

Fig. 1 Specific growth rate and ethanol production rate of *Kluyveromyces* sp. IIPE453 at **a** different temperatures and **b** pH: *filled circle* specific growth rate; *filled square* specific production rate

Table 1 Cell mass and ethanol yields on different sugars by *Kluyveromyces* sp. IIPE453 at 50°C

Sugar substrate	Sugar consumed (g l^{-1})	Dry cell weight (g l^{-1})	Biomass yield (% $Y_{X/S}$)	Sugar consumed (g l^{-1})	Ethanol conc. (g l^{-1})	Ethanol yield (% $Y_{P/S}$)
Glucose	10 ± 0.1	2 ± 0.1	20 ± 0.1	20 ± 0.1	9.2 ± 0.2	46 ± 0.7
Galactose	10 ± 0.1	2 ± 0.3	20 ± 0.3	20 ± 0.2	9.4 ± 0.2	47 ± 0.5
Mannose	10 ± 0.1	1.2 ± 0.2	12 ± 0.2	20 ± 0.2	6.6 ± 0.4	33 ± 1.6
Xylose	10 ± 0.1	4.3 ± 0.1	43 ± 0.05	6.5 ± 0.5	0.7 ± 0.06	10.7 ± 0.01
Arabinose	2.6 ± 0.2	0.15 ± 0.02	6.1 ± 0.3	0	0	–
Sucrose	10 ± 0.1	1.3 ± 0.05	13 ± 0.4	20 ± 0.1	10.1 ± 0.2	50.5 ± 0.5
Lactose	4.4 ± 0.1	0.98 ± 0.06	22.2 ± 0.8	10.2 ± 0.3	1.7 ± 0.2	16.6 ± 1.4
Cellobiose	5.6 ± 0.2	2 ± 0.1	35.5 ± 0.5	9.4 ± 0.4	0.4 ± 0.05	4.4 ± 0.2
Raffinose	5.5 ± 0.2	1.9 ± 0.1	34.5 ± 0.5	5.3 ± 0.1	0.6 ± 0.08	11.3 ± 1.2

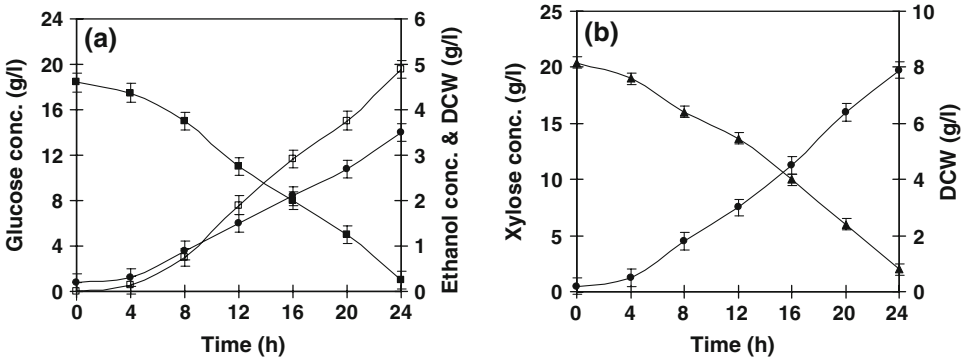

Fig. 2 Growth of *Kluyveromyces* sp. IIPE453 at 50°C on **a** glucose and **b** xylose: *filled square* glucose concentration, *filled triangle* xylose concentration, *filled circle* dry cell weight (*DCW*), *open square* ethanol concentration

The yeast *Kluyveromyces* sp. IIPE453 could ferment a glucose concentration of 200 g l^{-1} at 50°C with 85% sugar conversion to ethanol. The maximum ethanol concentration was 82 ± 0.5 g l^{-1} on initial glucose concentration of 200 g l^{-1} with ethanol yield of 46 ± 0.2% and productivity 1.71 ± 0.1 g l^{-1} h^{-1} in 48 h. The ethanol productivity increased 1.85 ± 0.1 and 1.93 ± 0.1 g l^{-1} h^{-1} when initial glucose concentrations were decreased 100 and 50 g l^{-1}, respectively (Fig. 3a). The strain could ferment xylose at different concentrations of 20–40 g l^{-1} at 50°C. The maximum ethanol concentration of 1.75 ± 0.05 g l^{-1} with ethanol yield of 10 ± 0.1% and productivity of 0.025 ± 0.005 g l^{-1} h^{-1} and maximum xylitol concentration of 11.5 ± 0.4 g l^{-1} with xylitol yield of 65 ± 2% and productivity of 0.17 ± 0.02 g l^{-1} h^{-1} were obtained (Fig. 3b).

In another study, the strain *Kluyveromyces* sp. IIPE453 could ferment the glucose and xylose mixture, achieving ethanol concentration of 38 ± 0.5 g l^{-1} with ethanol yield of 75 ± 0.9% of theoretical yield on total consumed sugar, and productivity of 0.79 ± 0.01 g l^{-1} h^{-1} and xylitol concentration of 14.5 ± 0.2 g l^{-1} with xylitol yield of 65.6 ± 1.2% on xylose consumed and productivity of 0.3 ± 0.01 g l^{-1} h^{-1} at 50°C (Fig. 4). In a continuous process of cell recycling at the dilution rate of 0.1 h^{-1}, the steady state was reached after 42 h. The maximum ethanol concentration of 30.8 ± 6.2 g l^{-1} with ethanol yield of 78.2 ± 3.8% of theoretical yield on total sugar consumed and productivity of 3.1 ± 0.6 g l^{-1} h^{-1} and xylitol concentration of 7.35 ± 3.3 g l^{-1} with xylitol yield of 63 ± 3% on xylose consumed and productivity of 0.75 ± 0.35 g l^{-1} h^{-1} were achieved in steady state (Fig. 5).

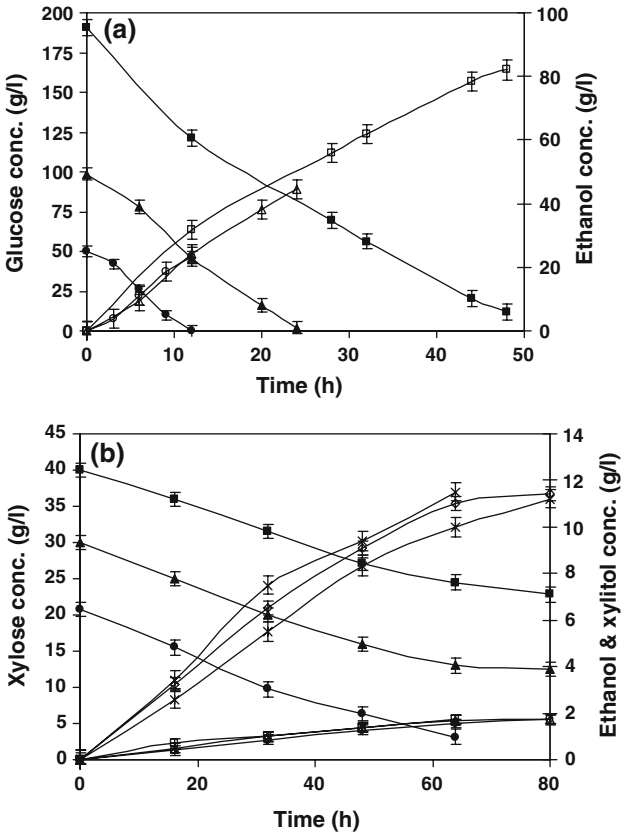

Fig. 3 Batch fermentation by *Kluyveromyces* sp. IIPE453 at 50°C **a** on glucose: glucose concentration *filled circle* $S_o = 50$ g l^{-1}, *filled triangle* $S_o = 100$ g l^{-1}, *filled square* $S_o = 200$ g l^{-1}; ethanol concentration *open circle* $S_o = 50$ g l^{-1}, *open triangle* $S_o = 100$ g l^{-1}, *open square* $S_o = 200$ g l^{-1}, **b** on xylose: xylose concentration *filled circle* $S_o = 20$ g l^{-1}, *filled triangle* $S_o = 30$ g l^{-1}, *filled square* $S_o = 40$ g l^{-1}; ethanol concentration *open triangle* $S_o = 20$ g l^{-1}, *open triangle* $S_o = 30$ g l^{-1}, *open circle* $S_o = 40$ g l^{-1}; xylitol concentration *multiplication sign* $S_o = 20$ g l^{-1}, *open diamond* $S_o = 30$ g l^{-1}, *asterisk* $S_o = 40$ g l^{-1}

Discussion

Table 1 shows the cell-mass yield and ethanol yield on different sugars by newly isolated yeast *Kluyveromyces* sp. IIPE453. The maximum cell-mass yield could be achieved on xylose, whereas the yield was low on arabinose. The yield on cellobiose was comparable with xylose. The hexose sugars, such as glucose, mannose, galactose, sucrose, and lactose, showed the lower yield of cell mass due to the formation of ethanol and other metabolites, such as acetaldehyde, acetic acid, lactic acid, acetone, ethyl acetate, and higher alcohols during growth [8, 18]. The maximum ethanol yield was achieved on sucrose, which was

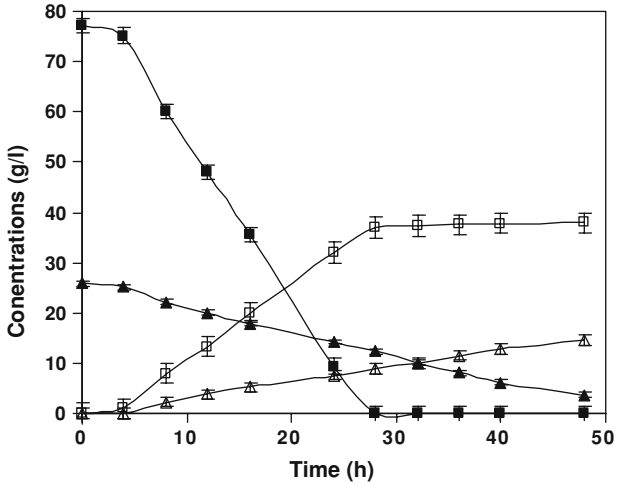

Fig. 4 Batch fermentation on glucose and xylose mixture by *Kluyveromyces* sp. IIPE453 at 50°C: *filled square* glucose concentration, *filled triangle* xylose concentration, *open square* ethanol concentration, *open triangle* xylitol concentration

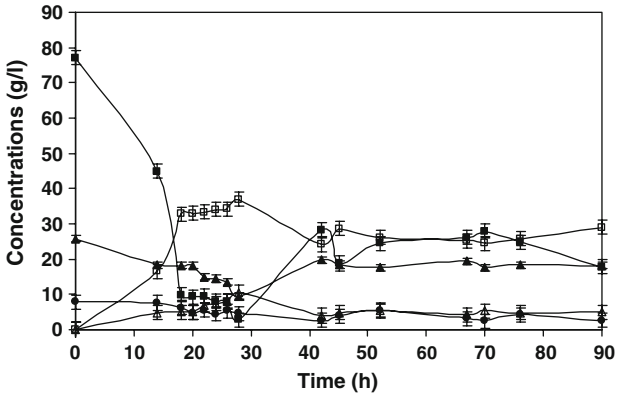

Fig. 5 Continuous fermentation by recycling the cells at a dilution rate of 0.1 h^{-1} on glucose and xylose mixture by *Kluyveromyces* sp. IIPE453 at 50°C: *filled square* glucose concentration, *filled triangle* xylose concentration, *filled circle* dry cell weight (*DCW*); *open square* ethanol concentration, *open triangle* xylitol concentration

around 50.5 ± 0.5% without any growth at 50°C, whereas Fleming et al. [9] reported 35% ethanol yield with 0.7 h^{-1} specific growth rate on sucrose by *Kluyveromyces marxianus* IMB3 at 45°C. The ethanol yield on hexoses, such as glucose and galactose, was 46 ± 0.7% and 47 ± 0.5%, respectively, whereas on mannose, the yield was 33 ± 1.6%. The yeast could also produce the ethanol on cellobiose with low yield and low sugar consumption. The strain showed the ethanol fermentation on xylose but could not ferment arabinose.

The strain has the ability to convert hexose sugars to cell mass as well as ethanol during the growth phase. This ability shows that the yeast follows the Crabtree rather than the Pasteur effect [5]. The yeast *Saccharomyces cerevisiae* also follows Crabtree [23]. Figure 2 shows the growth and ethanol production simultaneously with specific growth rate of 0.23 h^{-1} on glucose, whereas on xylose, the cells grew with specific growth rate of 0.34 h^{-1} without producing ethanol in aerobic conditions. Banat et al. [5] reported specific growth rate of *Kluyveromyces marxianus* IMB3 0.63 and 0.19 h^{-1} on glucose and xylose, respectively, in batch fermentation at 50°C.

The yeast *Kluyveromyces* sp. IIPE453 could ferment the glucose concentration 200 g l^{-1} within 48 h, achieving an ethanol yield of 90% of theoretical yield, with productivity of 1.71 ± 0.1 g l^{-1} h^{-1} and specific productivity of 0.38 ± 0.1 g g^{-1} h^{-1} in a batch process at 50°C (Fig. 3a), whereas the maximum ethanol productivity of 1.93 ± 0.1 g l^{-1} h^{-1} was obtained on initial glucose concentration of 50 g l^{-1}. The maximum ethanol concentration after 48 h was 8.2 ± 0.05% (w/v) on the initial glucose concentration of 200 g l^{-1} indicates high glucose and ethanol tolerance of the strain at 50°C. Banat et al. [3] reported the maximum ethanol concentration of 7.2% (w/v) with ethanol yield of 98% of theoretical yield and ethanol productivity 1.71 g l^{-1} h^{-1} on 140 g l^{-1} glucose by *Kluyveromyces marxianus* IMB2 at 45°C, whereas there was an ethanol concentration of 5.5% (w/v) with ethanol yield of 98% of theoretical yield and ethanol productivity of 1.31 g l^{-1} h^{-1} at 50°C by the same strain.

In fermentation with xylose, the maximum xylose concentration of 17.65 ± 0.05 g l^{-1} could be used. The yeast could ferment the xylose to an ethanol concentration of 1.75 ± 0.05 g l^{-1} with ethanol yield of 20 ± 0.3% of theoretical and ethanol productivity of 0.025 ± 0.005 g l^{-1} h^{-1}, as well as xylitol concentration of 11.5 ± 0.4 g l^{-1} with xylitol yield of 65 ± 2% and xylitol productivity of 0.17 ± 0.02 g l^{-1} h^{-1} at 50°C, as shown in Fig. 3b. Wilkins et al. [32] reported maximum xylose consumption of 13.61 g l^{-1} in 96 h with ethanol productivity of 0.02 g l^{-1} h^{-1} and xylitol productivity of 0.08 g l^{-1} h^{-1} using *Kluyveromyces marxianus* IMB4 at 40°C. In another study, Yablochkova et al. [33] reported ethanol productivity of 0.315 g l^{-1} h^{-1} on glucose and ethanol productivity of 0.0033 g l^{-1} h^{-1} and xylitol productivity of 0.105 g l^{-1} h^{-1} on xylose by *Kluyveromyces marxianus*. No significant change was observed during fermentation in the cell-mass concentration, which means the ethanol formation is non-growth associated when using *Kluyveromyces* sp. IIPE453.

Figure 4 shows the ethanol fermentation in batch on the mixture of glucose and xylose simultaneously by *Kluyveromyces* sp. IIPE453 at 50°C. The glucose consumption rate (3.8 ± 0.05 g h^{-1}) was higher than that of xylose (0.73 ± 0.03 g h^{-1}). Glucose was consumed within 28 h, but the total xylose was consumed in 48 h. The strain was able to use glucose and xylose simultaneously, which has not thus far been reported when using *Kluyveromyces* sp.

In a continuous process with recycling the cells at 50°C after achieving the steady state, the maximum ethanol concentration of 37 ± 0.1 g l^{-1} with ethanol yield of $82.2 \pm 0.2\%$ of theoretical yield could be produced with volumetric productivity of 3.7 ± 0.01 g l^{-1} h^{-1} and specific productivity of 0.8 ± 0.03 g g^{-1} h^{-1}, besides $65.6 \pm 0.2\%$ xylitol yield on consumed xylose basis with xylitol productivity of 1.1 ± 0.08 g l^{-1} h^{-1} and specific productivity of 0.24 ± 0.03 g g^{-1} h^{-1} (Fig. 5). Cell concentration was declining due to partial cell recycling in the absence of any cell growth. However, the specific ethanol productivity (0.6–0.8 g g^{-1} h^{-1}) remained the same during fermentation, indicating the high stability of cells to 90 h.

Conclusions

The new isolated thermotolerant yeast strain *Kluyveromyces* sp. IIPE453 (MTCC 5314) has shown the consumption of a wide range of sugars, which are the major constituents of lignocellulosic biomass either for growth or ethanol fermentation. The strain showed the simultaneous uptake of glucose and xylose for ethanol and xylitol production with high productivity. It also showed an ethanol tolerance up to 8.2% (w/v). The study revealed that the characteristics of the yeast strain allow it to grow efficiently on xylose and ferment glucose efficiently to ethanol. Such characteristics have the potential to develop a bioprocess in which the xylose part of the lignocellulosic biomass can be used to grow the strain as well as produce xylitol, and the glucose portion can be used for ethanol production.

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