Isolation and characterization of a non-*Saccharomyces* yeast with improved functional characteristics for ethanol production

Abstract

Out of the twenty yeasts isolated from over-ripened fruits and naturally fermenting sugarcane juice, four isolates showing relatively higher fermentation ability were screened for their fermentation potential. Isolate Y-4 produced relatively higher ethanol than the other isolates from 15 gl^{-1} glucose. The cells of isolate Y-4 produced ascospores. Scanning electron micrographs (SEM) of isolate Y-4 showed oval to spherical cells with diameter ranging from 4.5 to 6.2 µm. On the basis of the SEM images and 28s rRNA gene sequencing, isolate Y-4 was identified as *Pichia kudriavzevii* and designated as *P. kudriavzevii* SK1. *Pichia kudriavzevii* SK1 metabolized glucose, galactose, mannose, maltose and fructose. It showed the potential to grow at a glucose concentration of 300gl^{-1} and ferment at 45 ˚C, though the best results were observed at 15-20 gl^{-1} glucose at 35 ˚C. Reference strain *Saccharomyces cervisiae* MTCC 11815 produced low concentrations of ethanol under similar conditions. With 200 gl^{-1} initial glucose concentration 86.1 and 87.9 gl^{-1} ethanol was obtained in shake flasks and laboratory batch fermenter experiments, respectively at pH 5 at 35 ˚C. This study revealed that *P. kudriavzevii* SK1 could be utilized for pilot scale fermentation for high gravity fermentations.

Keywords: Ethanol productivity; Glucose concentration; *non-Saccharomyces* yeasts; *Pichia kudriavzevii*;Thermotolerant yeasts

1. Introduction

Yeasts, in particular *Saccharomyces cerevisiae* have been used since ancient times in brewing, alcohol production and baking processes (Lee et al, 2003). From the commercial perspective, a strain capable of tolerating high ethanol and sugar concentrations and possessing invertase activity is desirable (Osho et al, 2010), especially in high gravity (VHG) fermentations which are common in the ethanol industry wherein the yeast cells are subjected to tolerate high sugar concentrations at the beginning of the fermentation process and high ethanol concentration at the end of the process (Tikka et al, 2013).

Non-*Saccharomyces* yeast strains have normally been excluded from fermentation due to production of spoilage metabolites, off odours and low fermentative ability (Chatonnet et al, 1995). Some non-*Saccharomyces* yeast species have also been reported to improve the fermentation behaviour of yeast starter cultures and the analytical composition of wine, in terms of more complex aroma (Esteve-Zarzoso et al, 1998; Heard, 1999). Many non- *Saccharomyces* strains have been commercialized and are available in ethanol (Dhaliwal et al, 2011), beer (Tataridis et al, 2013)and wine making.

Even though *Saccharomyces* spp. are being used for alcoholic fermentation but due to limitations such as, low sugar and ethanol tolerance and fermentative ability at elevated temperatures non-*Saccharomyces* spp. have been used. *Klyuveromyces* and *Pichia* spp. have the potential to ferment sugars at higher temperatures (≥ 40 °C) and tolerate high ethanol concentration. (Oberoi et al, 2011). A thermotolerant *Pichia*

kudriavzeii strain produced 35.5 g/l and 33.8 g/l ethanol (Chamnipa et al, 2018) at 37 °C and 40 °C, respectively.

A strain that produces a favourable metabolite, thereby enhancing the quality of final product can be selected for industrial application (Ciani et al, 2010). Therefore, the present work was designed to isolate, identify and characterize the non-*Saccharomyces* strain to assess it ethanol potential for laboratory and pilot scale.

Materials and methods

2.1 Materials

The yeast samples were isolated from over-ripened grapes, apples, pears and naturally fermenting sugarcane juice were used for isolation of yeasts. Standards for sugars (glucose, fructose, sucrose, xylose, arabinose, galactose and rhamnose) were prepared during the HPLC determination were procured from Sigma- Aldrich (St. Louis, MO, USA) While chemicals used during analytical work and preparation of dehydrating media were procured from Fisher Scientific (Mumbai, India) and Hi-Media Laboratories (Mumbai, India), respectively. Carbohydrate assimilation capacity of cells to metabolize different sugars and urease activity were analyzed using KB009 Hi-Carbohydrate and KB006 Hi-Candida kits, respectively (Hi-Media Laboratories Pvt. Ltd, Mumbai, India). *Saccharomyces cerevisiae* MTCC 11815 was procured from Department of Microbiology, Punjab Agriculture University, Ludhiana, Punjab, India.

2.1.1 Analytical methods

Yeast cell count was estimated with a haemocytometer (Hausser Sci., USA) and the cell viability was assessed by staining the cells with 0.1% methylene blue solution (Borzani and Vario, 1958).Reducing sugars were determined by the

dinitrosalicylic acid (DNS) method (Miller 1959).Glucose and ethanol were determined with HPLC [Ultimate 3000, Dionex Corporation, Sunnyvale, CA, USA] (Oberoi et al (2012) using RI detector and quantified based on the area and retention time of the standards.

2.1.2 Isolation of yeasts

The yeast cultures were isolated from rotten fruits viz. apple, grapes and pears. The fruits were allowed to rot at room temperature, and the extracted juice was collected in sterile containers aseptically. The juice was appropriately diluted and plated on glucose yeast peptone agar (YPD). YPD medium containing (gl^{-1}) glucose (20), peptone (10), yeast extract (5), and agar (15) was used to isolate yeasts by pour plate method. Initial pH of the medium was adjusted to 5.0 with 5 N HCl or NaOH. Morphologically, characteristic yeast colonies were picked; cultures were purified by streaking and preserved on YPD agar slants. The potential for ethanol production of selected isolates was tested in a medium containing (gl^{-1}) glucose (50), peptone (20), yeast extract (20) and $MgSO_4$ (20) at pH 5 (Dhaliwal et al, 2011). All the flasks were incubated at 30 °C in an incubator shaker which was maintained at 100 rpm. Samples were periodically drawn at 6 h interval till 48 h and analysed for ethanol and glucose concentrations. Fermentation efficiency of the isolates was calculated in terms of ethanol produced.The selected isolates were further compared for their ethanol producing abilities at relatively higher glucose concentration of 150 gl^{-1} at 35 °C while maintaining the pH at 5. The temperature was raised from 30 to 35 °C with the aim of isolating a thermotolerant strain.

2.1.3 Identification of the screened yeast isolate

The screened and selected yeast isolate was grown for 24 h at 30 °C in 50 ml YPD broth and DNA was extracted (Harju et al, 2004). The D1/D2 region of the large sub-unit (LSU) of the 28S rDNA region was amplified with PCR using forward primer 5'ACCCGCTAACTTAAGC3' and reverse primer 3'GGTCCGTGTTTCAAGACGG5'. The PCR amplified products were then purified (Qiagen Mini elute Gel extraction kit) and sequenced (ABI 3730xl Genetic Analyzer (Applied Biosystems, USA; Sandhu et al, 2012).

2.1.4 Biochemical and microscopic characterization of selected yeast isolate

Resistance to 1% acetic acid, 0.01% and 0.1% cycloheximide was assessed by incorporation of acetic acid and cycloheximide at concentrations mentioned above in the sterilized YPD broth flasks that were inoculated with the selected strain. Flasks were incubated at 30 ºC for 24 h (200 rpm). All the experiments were performed in triplicates (Dhaliwal et al, 2011). The scanning electron microscope studies were performed as per Bozolla and Russell (1994).

2.2 Comparative evaluation of ethanol production

Fermentative ability of *P. kudriavzevii* SK1 cells was compared with that of *S. cerevisiae* MTCC 11815 cells in ethanol production using synthetic medium. Inoculum preparation and incubation conditions remained same as described previously for *S. cerevisiae* MTCC 11815 and *P. kudriavzevii* SK1. Flasks containing 150 ml fermentation medium composed of (gl^{-1}) glucose (150), yeast extract (2), peptone (2) and $MgSO_4$ (2). Inoculum (10 ml) having 1×10^8 cells/ml was used. Experiments were performed using one factor at a time approach with glucose

concentration varying between 100-300 gl^{-1}, pH varying between 3 to 6 and temperature ranging from 25-45 °C.

2.3 Ethanol production by *P. kudriavzevii*

On the basis of the comparative evaluation results, an experiment was performed with *P. kudriavzevii* SK1 at 200 gl^{-1} glucose concentration (obtained by saccharification of rice carried out at 20% substrate in water, pH 5.3, temperature 55˚C and enzymes, 30 IU/g α-amylase and 50 IU/g glucoamylase (standardized on basis of preliminary trials in our laboratory), initial pH and incubation temperature of 5.0 and 35 ˚C, respectively at 200 rpm. Samples were drawn regularly at 6 h interval up to 60 h and analyzed for glucose and ethanol concentrations. All the experiments were conducted in triplicates and the data were analyzed statistically.

2.4 Ethanol production in laboratory in fermenter

On the basis of the preliminary results, ethanol production was carried out in 2.5 L batch reactor (Minifors, Infors HT, Switzerland). About 1600 ml broth containing 200 gl^{-1} glucose (obtained by sachharification of rice as described previously) was supplemented with 2 gl^{-1} yeast extract, 2gl^{-1} peptone and 2 gl^{-1} $MgSO_4.H_2O$. After sterilization and cooling, pH of the medium was adjusted to 5.0 with the sterilized 5 N HCl solution and the medium after cooling was inoculated with 10% (v/v) yeast cells at a cell concentration of 1×10^8 cells/ml. Agitation, pH and temperature were maintained at 100 rpm, 5.0 and 35˚C, respectively throughout the fermentation process. Samples were drawn regularly at 6 h intervals up to 60 h and analysed for glucose and ethanol concentrations. The experiment was conducted three times in the same fermenter and results were statistically analysed.

3. Results & Discussions

Twenty yeast isolates were selected on the basis of microscopic examination and their pure cultures were maintained on YPD – agar slants. Selected isolates were tested for different characteristics, such as growth in presence of 5 gl^{-1} ethanol and ethanol production potential. Seventeen isolates were able to grow at 30 °C in presence of 5 gl^{-1} ethanol but only 13 could ferment glucose to ethanol. Out of the 13 isolates, four isolates, Y-4, Y-6, Y-10 and Y-15 showed characteristic diversity in terms of colony and cell morphology and also high cell count in the range of 1×10^{8}cells/ml or more in 48 h as compared to the remaining isolates. Y-4, Y-6, Y-10 and Y-15 produced 23.1, 22.0, 22.7 and 21.0 gl^{-1} ethanol (Table 1), respectively from an initial 50 gl^{-1} glucose concentration, which was relatively higher (>80% fermentation efficiency) than the other isolates that showed capability to ferment glucose to ethanol in presence of ethanol. On the basis of final ethanol concentration obtained, the above four isolates were selected for further studies.

3.1 Ethanol production by the selected yeast isolates

To select the strain which showed highest fermentation efficiency, the four yeast isolates were further compared for their ethanol producing abilities at relatively higher glucose concentration of 150 gl^{-1} and temperature of 35 °C. Isolate Y-4 produced 68.0 gl^{-1} ethanol showing highest fermentation efficiency of 88.92% as compared to the other three isolates. Ethanol production levelled off after 36 h for all the four isolates corresponding to ethanol productivity of 1.9, 1.7, 1.7, 1.4 g/L/h for isolates Y-4, Y-6, Y-10 and Y-15, respectively (Table 1). High glucose consumption and ethanol yield are known to be indicators of osmotolerance by yeasts (Favaro et.

al. 2013). On the basis of high ethanol producing ability isolate Y-4 was selected for further fermentation studies.

3.2 Identification of isolate Y-4

Sequencing and analysis of the 28s rRNA region of the yeast strain revealed that this region had the highest identity with *I. orientalis* F701. Phylogenetic relationships were drawn using the alignment and cladistic analysis of homologous nucleotide sequences of known microorganisms. The isolated yeast strain belonged to the same branch as *I. orientalis* F701with 100% homology in the 28s rRNA region. On the basis of the morphology and the comparison of 28s rRNA gene sequence, the isolated yeast strain was confirmed *P. kudriavzevii* and was designated as *P. kudriavzevii* SK1. The 28s rRNA gene sequences for *P. kudriavzevii* were submitted to GenBank with accession number JX537791.1. The species ascribed to genus *Issatchenkia* has been clustered within *Pichia*, and thus all isolates of *Issatchenkia* are replaced with *P. kudriavzevii* for taxonomic entity (Kurtzman 2008, Oberoi et al, 2010)

3.3 Biochemical and microscopic characteristics of the selected isolate

Pichia kudriavzevii SK1 cells were able to utilize maltose, fructose, dextrose, galactose and mannose, but were unable to use xylose, raffinose, sucrose, trehalose, arabitol as carbon source. Microscopic observations revealed that the yeast cell produced ascospores. The isolate could not grow in presence of cycloheximidine and lacked lipolytic activity and was unable to metabolize starch.

3.4 Comparison of ethanol production by *P. kudriavzevii* SK1and *S.cerevisiae* MTCC11815

Initial sugar concentration is known to have a detrimental effect on fermentation performance, effecting yeast physiology and altering the physical and flavour properties.It was observed that final ethanol concentration obtained after 60 h fermentation increased with increase in initial substrate concentration from 100 to 200 gl^{-1} for two strains at pH 5 and 35 °C (Table 2). However, ethanol production rate decreased at 250 and 300 gl^{-1} glucose concentrations during fermentation, though ethanol concentration was found to be higher from higher glucose concentrations at the end of fermentation period.The fermentation efficiency decreased from 96.5% to 77.28% as initial sugar concentration increased from 100 gl^{-1} to 300 gl^{-1}. *P. kudriavzevii* SK1 produced about 20% higher ethanol as compared to *S. cerevisiae* MTCC11815 after 48 h of fermentation. It is noteworthy to mention here that the time taken to complete fermentation was 60 h with initial glucose concentration of 30 gl^{-1}, while fermentation could be completed in 48 h at initial glucose concentration of 20 gl^{-1} or less.

The two strains were tested for ethanol fermentation ability with changes in pH (3-6) (Table 2) from 200 gl^{-1} initial sugars and35°C. Significant difference in ethanol production was not seen at pH of 3 or 3.5 for either of the isolates with *P. kudriavzevii* SK1 producing a slightly higher ethanol than *S. cerevisiae*. Both the stains showed maximum ethanol production at pH 5 with *P. kudriavzevii* SK1 producing about 22% more ethanol than *S. cerevisiae* MTCC11815.

It was observed that similar ethanol concentration at temperatures of 25 and 30 ˚C (initial sugars 200 gl^{-1} pH 5) but as the temperature increased, efficiency of *S. cerevisiae* MTCC11815 decreased and it could produce only 53 gl^{-1} ethanol at 35 °C with ethanol concentration further declining to 20.9 g/L and 10 g/L at 40 and 45 °C respectively (Table 2). *P. kudriavzevii* SK1 however produced ethanol in concentrations ranging between 80-92 g/L at temperatures ranging from 25-35 °C. These results confirmed that *P. kudriavzevii* SK1 showed higher fermentation efficiency than *S. cerevisiae* MTCC11815 at 35 °C. *P. kudriavzevii* SK1 produced 60.4 gl^{-1} and 48 gl^{-1} ethanol at 40 and 45 ˚C, respectively in 48 h, which were nearly three times higher than the ethanol concentration at 40 °C and about five times higher at 45 °C produced by the standard reference isolate. Even at 35 °C, ethanol concentration after 48 h was twice for *P. kudriavzevii* SK1 as compared to *S. cerevisiae* MTCC11815. However, in most of the cases, ethanol concentration leveled off after 48 h which could be because of the depletion of nutrients, stress due to prolonged growth and production of certain toxic metabolites in the medium. Techaparin et al (2017) isolated *P. kudriavzevii*strains which were able to ferment ethanol upto 45 °C. Yuansgard et al (2013) obtained 70.6 gl^{-1}ethanol at 40 °C from 180 gl^{-1} initial sugarsusing *P. kudriavzevii* isolates.

3.5 Ethanol production by *P. kudriavzevii* SK1 in a laboratory fermenter

In shake flasks and in laboratory fermenter 86.1 and 87.9 gl^{-1} ethanol concentration (Figure1), (Figure 2) was obtained at 48 h.The respective fermentation efficiencies were 93.7 and 95.75 % and ethanol productivity at 48 h in shake flasks and batch fermentor were 1.79 and 1.83 g/L/h, respectively . Although no significant difference in ethanol concentration was observed in shake flask and laboratory

fermenter, higher ethanol productivity was obtained with laboratory fermentor. Ethanol production rate decreased after 36 h of fermentation and leveled off after 48 h.The results are in consonance with the previously reported results (Oberoi et al, 2012) wherein higher ethanol concentration in laboratory fermenter was reported compared to shake flasks under the same set of conditions. Kaewkrajay et al (2014) reported ethanol concentration of 42.4 gl^{-1} after 48 h at 45 ˚C using a thermotolerant strain of *P. kudriavzeii* in a 7 l jar fermenter. This study showed that *P. kudriavzevii* SK1 could be used in higher scale of operation.

Conclusion

P. kudriavzeii SK1, a non *Saccharomyces cerevisiae* strain showed the potential to ferment sugars at substrate concentration (100-250 gl^{-1}), pH (4.5-6.5) and temperature (25-45 °C) giving best results at 200 gl^{-1} substrate concentration, pH 5 and temperature 35°C. From 200 gl^{-1} glucose at 35 °C, *Pichia kudriavzevii* SK1 produced 87.9 gl^{-1} ethanol with a fermentation efficiency of about 91% in a laboratory fermenter resulting in a volumetric productivity of 1.8 g/L/h, thereby showing potential for commercial exploration.

Acknowledgement

Authors Kaur and Phutela thankfully acknowledge the financial assistance received from Punjab Agricultural University, Ludhiana and University Grants Commission under Rajiv Gandhi National Fellowship for SC & ST students. Grant No. 16-1192-(SC)/2008. Dated: 27.3.2009. Author Oberoi thankfully acknowledges the financial assistance received under AMAAS project from ICAR-NBAIM, Manju Nath Bhanjan, UP, India (NBAIM/AMAAS/2008-09/AMBPH-05/HSO/BG/3/5982).

Conflict of interest: NONE

Ethical Statement

'This article does not contain any studies with human participants or animals performed by any of the authors."

Author's Contribution

Authors Rajpal Phutela and Harinder Singh Oberoi designed the study being the major and minor guides of Doctorate program of author Sandeep Kaur. Author Sandeep Kaur conducted the study and wrote the manuscript. Author Harinder Singh Oberoi edited and contributed to the writing of the manuscript. All authors have read and approve the manuscript.

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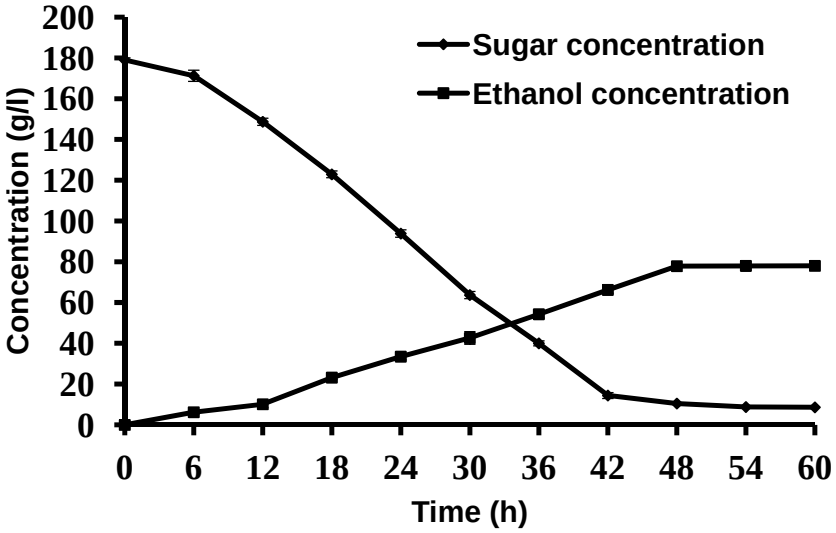
Table 1: Ethanol production by yeast isolates.

Isolate	Ethanol			
	Concentration (gl^{-1})	**Efficiency (%)**	**Concentration (gl^{-1})**	**Efficiency (%)**
Y-4	23.08	90.50	68.03	88.92
4-6	21.96	86.11	59.4	77.64
Y-10	22.66	88.86	62.64	81.88
Y-15	21.03	82.47	51.84	67.76
	C.D (5%) = 3.59		C.D (5%) = 1.73	
	Error = ±0.44		Error = ±0.52	
	Initial sugar concentration: 50 gl^{-1}		Initial sugar concentration: 150 gl^{-1}	
	Temperature: 30 °C		Temperature: 35 °C	
	pH: 5		pH: 5	

Table 2: Ethanol produced by *P. kudriavzevii* SK1 and *S.cervisiae* MTCC 11815

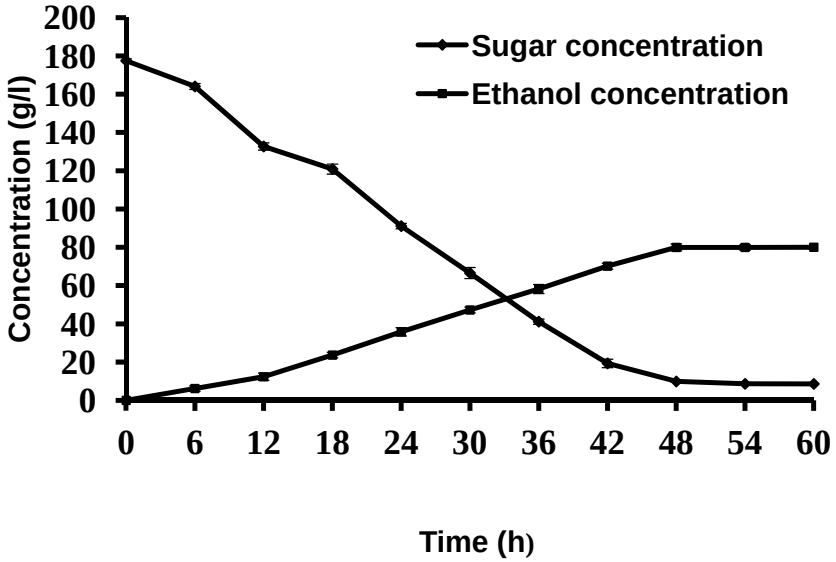
Glucose (gl^{-1})	**Ethanol (gl^{-1})**		**pH**	**Ethanol(gl^{-1})**		**Temp (°C)**	**Ethanol(gl^{-1})**	
	P. kudriavzevii SK1	*S.cervisiae* MTCC 11815		*P. kudriavzevii* SK1	*S.cervisiae* MTCC 11815		*P. kudriavzevii* SK1	*S.cervisiae* MTCC 11815
100	49.24 (48)	48.96 (48)	**3.0**	23.47 (48)	23.17 (48)	**25**	77.98 (48)	64.23 (48)
150	69.73 (48)	65.63 (60)	**3.5**	32.23 (48)	26.88 (48)	**3.**	76.93 (48)	65 (54)
200	90.62 (54)	88.83 (54)	**4.0**	39.81 (48)	35.24 (48)	**35**	65.47 (48)	53.34 (60)
250	104.83 (54)	90.08 (54)	**4.5**	48.6 (60)	43.06(60)	**40**	60.59 (48)	23.96 (48)
300	118.24 (54)	102.64 (54)	**5.0**	79.4 (54)	65.4 (48)	**45**	43.8 (60)	9.64 (54)
			5.5	78.51 (54)	64.28 (48)			
			6.0	75.86 (54)	60.14 (48)			
C.D.	**0.04**	**0.027**		**0.003**	**0.022**		**0.030**	**0.012**
SE(m)	**0.01**	**0.009**		**0.001**	**0.007**		**0.009**	**0.004**
SE(d)	**0.01**	**0.012**		**0.001**	**0.010**		**0.013**	**0.005**
C.V.	**0.026**	**0.019**		**0.003**	**0.027**		**0.025**	**0.015**
Temperature = 35 °C			Initial substrate concentration = 200 gl^{-1}			Initial substrate concentration = 200 gl^{-1}		
pH= 5			Temperature = 35 °C			pH= 5		

Values in brackets indicate the time taken in hours to obtain the highest ethanol production.



Initial glucose/sugar concentration: 180 gl^{-1} (Obtained from saccharification of 20% rice); pH: 5; Temperature: 35 °C

Figure 1: Ethanol production and glucose consumption by *Pichia kudriavzevii* SK1 in shake flasks



Initial glucose/sugar concentration: 180 gl^{-1} (Obtained from saccharification of 20% rice); pH: 5; Temperature: 35 °C

Figure 2: Ethanol production and glucose consumption by *Pichia kudriavzevii* SK1 in laboratory fermenter.