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A NEW MULTI-STRESS RESISTANT *Wickerhamomyces anomalus*: ISOLATION, IDENTIFICATION AND BIOETHANOL FERMENTATION POTENTIAL

UN NUEVO ANOMALISMO DE *Wickerhamomyces* RESISTENTE A MÚLTIPLES ESTRÉS: POTENCIAL DE AISLAMIENTO, IDENTIFICACIÓN Y FERMENTACIÓN DE BIOETANOL

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Abstract

Sustainable bio-renewable energy source is the need of hour to meet the rising global energy demands. Efficient conversion of fermentable sugars by yeast into bioethanol from lingo-cellulosic biomass is one of the most important attributes. In order to get temperature and ethanol tolerant yeast strain, one hundred and seventy one soil samples were collected from adjacent areas of various sugar mills located in Punjab, Pakistan. Fifty six yeast strains were isolated and primarily screened for bioethanol production. Maximum bioethanol producing (3.07±0.05 g/L) yeast strain IHZ-26 was identified by sequencing of 18S rRNA ITS region and identified as a new strain of *Wickerhamomyces anomalus* which was assigned GenBank accession number KT883963. This strain demonstrated substantial tolerance to ethanol (25%) and temperature (50 ºC) and have the ability to significantly ferment several sugars i.e. glucose, fructose, mannose, maltose, xylose and lactose. To the best of our knowledge, this is first report of multi stress tolerant *Wickerhamomyces anomalus* from Pakistan. This yeast with these distinct characteristics can be employed in bioethanol industry for more viable and economical process.

Keywords: Thermo-tolerant, yeast, biofuel, ethanol tolerant, bioenergy.

Resumen

La fuente de energía bio-renovable sostenible es la necesidad de una hora para satisfacer las crecientes demandas mundiales de energía. Uno de los atributos más importantes es la conversión eficiente de azúcares fermentables por levadura en bioetanol a partir de biomasa lingo-celulósica. Para obtener la temperatura y la cepa de levadura tolerante al etanol, se recolectaron ciento setenta y una muestras de suelo de áreas adyacentes de varios ingenios azucareros ubicados en Punjab, Pakistán. Se aislaron cincuenta y seis cepas de levadura y se seleccionaron principalmente para la producción de bioetanol. La cepa IHZ-26 máxima de levadura que produce bioetanol (3.07 ± 0.05 g/L) se identificó mediante la secuenciación de la región ITS del ARNr 18S y se identificó como una nueva cepa de *Wickerhamomyces anomalus* a la que se asignó el número de acceso de GenBank KT883963. Esta cepa demostró una tolerancia sustancial al etanol (25%) y temperatura (50 ºC) y tiene la capacidad de fermentar significativamente varios azúcares, es decir, glucosa, fructosa, manosa, maltosa, xilosa y lactosa. Según nuestro conocimiento, este es el primer informe de *Wickerhamomyces anomalus* de Pakistán, tolerante al estrés múltiple. Esta levadura con estas características distintas se puede emplear en la industria del bioetanol para un proceso más viable y económico.

Palabras clave: Termo tolerante, levadura, biocombustible, etanol tolerante, bioenergía.

1 Introduction

Environmental, economical and geopolitical concerns regarding the sustainability of fossil fuels have drawn the attention of scientists from all over the world to carry out research on bio-based alternative renewable energy resources, to overcome future energy crisis (Marques *et al*., 2018).

One of the most appropriate sources of renewable energy is bioethanol, since it is an eco-friendly oxygenated fuel and holds the capability to induce remarkable reduction in the increasing emission levels of the hazardous gases (Walter *et al*., 2018). As a matter of fact, several steps are involved in the successful conversion of lignocellulosic biomass into bioethanol.

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The process includes pretreatment of biomass, production of recombinant cellulase enzymes through a chain of procedures, saccharification, and conversion of sugars to ethanol. Additionally, the conversion of lignocellulosic hydrolysate into bio-ethanol by employing effective yeast strain is a hallmark in bioethanol production and is also the area of prime interest nowadays (Amani *et al*., 2017). Moreover, Saccharomyces cerevisiae (Brewer's yeast) is well known for its ability to convert fermentable sugars, particularly hexoses to ethanol, and is therefore employed widely in industries (Cunha *et al*., 2017). However, certain limitations are associated with this process involving yeast strain; such as, the inability to ferment hexoses and pentoses simultaneously with the same efficiency; the factor of high temperature and ethanol tolerance, and the inhibition of process by components produced during break down of lignocellulosic biomass. As a consequence, scientists now look forward to an alternative strategy to overcome these inadequacies (Althuri *et al*., 2018). This has led them to search for an efficient source of ethanol and a temperature tolerant yeast strain with capability of fermenting multiple sugars simultaneously (Flores *et al*., 2018).

For the identification of yeast species, ITS (Internal Transcribed Spacer) region and 26S DNA (D1/D2 regions) are considered by employing ribotyping technique. Comparative assessment of ITS regions can produce better results in the identification of closely related species as compared to other rRNA sequences (Nilsson *et al*., 2015). For isolation of such potent sugar fermenting yeast, two parameters are usually considered, namely temperature and ethanol tolerance, as they both contribute to the economics of the process. Currently, some industries are carrying out ethanol fermentation using yeast at 30-34 ºC and maintenance of this specific temperature involves a continuous cooling process. Moreover, summer season is a challenge to this requirement. Therefore, industrial units prefer the use of thermotolerant yeast in order to conduct ethanol fermentation at elevated temperatures, thus eliminating the need of cooling systems (Khatun *et al*., 2017). Carrying out fermentation at high temperature not only increases the rate of process, but also makes it comparatively more economical by eliminating the need of continuous cooling for fermentation process (Hoshida and Akada, 2017).

Multiple genes were reported to express excessively in ethanol tolerance of yeasts. Usually these genes are found expressive during the dormant phase of yeast cells. Rarely, these genes are found active during the exponential phase of yeast cell growth (Stanley et al, 2010). Such yeasts possess the ability of ethanol and many other stresses tolerance. The ethanol-tolerant yeasts are usually found resistant to other stresses as well which include heat, high osmolarity, oxidative stress and pH (Ogawa *et al*., 2000; Jansen *et al*., 2017). Additionally, the ethanol tolerant yeast enhances the activity period of the reaction thus making the process more productive (Dalawai *et al*., 2017).

In the current study, isolation of multi-stress tolerant yeast (*Wickerhamomyces anomalus*) was carried out from the soil samples collected from an area adjacent to a sugar mill in Jhang, Punjab, Pakistan. To analyze the fermentation of multi-saccharides simultaneously, this yeast was assessed for its ability to convert different fermentable saccharides, along with its tolerance level against stress.

2 Materials and methods

2.1 Sample collection

Soil samples were collected from surrounding area of different sugar industries in Punjab, Pakistan which include Haseeb Waqas Sugar mill, Jhang; Pattoki Sugar mills, Pattoki and Ashraf Sugar mills, Bahawalpur. Samples were collected from a depth of 4 inches and kept in sterilized zipper polythene bags before transfer to laboratory (Muthukumaran, 2017).

2.2 Isolation of thermophilic yeast

Soil samples were serially diluted using normal saline solution. Serial dilution of 10-5 and 10-7 were poured (0.1 mL) onto YEPDA medium plates aseptically. Plates were incubated for 24h at 45 ºC to get the isolated colonies. After 24h plates were observed for yeast growth, transferred on YEPDA slants and incubated at 45 ºC for 24h. After growth, slants were stored at 4 ºC for further use (Dalawai *et al*., 2017).

2.3 Screening for ethanol production

The isolated strains were screened for their ethanol production ability. Fermentation was carried out after Shaghaghi *et al*. (2017) in YPD broth. Potassium Dichromate test was performed for ethanol production according to the method of Gupta *et al*., (2011). Estimation of residual sugar was carried out using DNS method (Miller, 1959).

2.4 *Scanning Electron Microscopy*

Maximum ethanol producing yeast strain IHZ-26 was sent to the Center for Advance Studies in Physics (CASP), Government College University Lahore, Pakistan for scanning electron microscopy. Electron micrographs of the samples were taken at different magnifications (50×).

2.5 *Molecular identification*

The yeast strain IHZ-26 was subjected to DNA isolation and PCR amplification after the using method of Nilsson *et al*. (2015). PCR products were subjected to 1% agarose gel electrophoresis at a constant voltage of 80 V for 30 min using 250-10000 bp DNA ladder. The gel was UV visualized. The targeted DNA size was sequenced using ITS region and sequence obtained was analyzed for percentage identity using BLAST. Fifteen closely related blast hits were used for multiple sequence alignment using the software Clustal W2. Aligned multiple sequences were used to develop dendogram by using software PHYLIP to distinguish the closely related species (Haq *et al*., 2014).

2.6 *Inoculum preparation*

A 24 h old *Wickerhamomyces anomalus* IHZ-26 culture was used to prepare inoculum for further fermentation experiments. A loop full of yeast was transferred to 25 ml Potato dextrose broth aseptically and incubated overnight at 45 ºC and 150 rpm. After incubation, broth was centrifuged and yeast pellet thus obtained was used for further inoculation (Abdul *et al*., 2017).

2.7 *Ethanol tolerance*

Ethanol tolerance capability of *Wickerhamomyces anomalus* IHZ-26 was determined following the method of Oshoma *et al*. (2015) with modification. YPD media (50 ml) were prepared with variable concentration of absolute ethanol (5, 10, 15, 20 and 25% (v/v) in 100 ml culture bottles with carbon dioxide outlet and sterilized. Each culture bottle was inoculated with 0.5g (wet weight) 24h old freshly prepared yeast pellet. Culture bottles were incubated at 45 ºC for 7 days at 150 rpm. Viability of cells was analyzed using haemocytometer (Marienfeld, Germany) and methylene blue.

2.8 *Thermo tolerance*

Thermo-tolerance of *Wickerhamomyces anomalus* IHZ-26 was assessed following the method of Patel *et al*., (2017) with modification. Sterilized YPD medium (50 mL) in 100 ml culture bottles with carbon dioxide outlet were inoculated with 0.5 g (wet weight) 24h old freshly prepared yeast pellet. Culture bottles were incubated at different temperatures: 45, 50, 55 and 60 ºC for 7 days in a shaking incubator (Vision Scientific Co. VS-8480, Korea) at 150 rpm. Viability of cells was analyzed using haemocytometer and methylene blue (Oshoma *et al*., 2015).

2.9 *Multiple sugars utilization*

Wickerhamomyces anomalus IHZ-26 was analyzed for its ability to convert various carbon sources into ioethanol. YPD medium (50 ml) containing 2% either of different carbohydrates (Glucose, Fructose, Sucrose, Maltose, Mannose, Lactose and Xylose) were prepared in separate culture bottles. Each culture bottle was inoculated with 0.5g (wet weight) 24 h old freshly prepared yeast inoculums pellet and incubated at 50 ºC and 150 rpm (Dorado *et al*., 2017).

2.10 *Statistical analysis*

SPSS (VERSION 16.0.) was used for carrying out the statistical analysis of the results. Significant difference with the probability (P) value was observed by applying one way ANOVAs on replicates. Y-error bars in figures indicate the standard deviation (±SD) among the three parallel replicates which differ significantly at $P \leq 0.05$.

3 Results and discussion

3.1 *Isolation and screening of thermophilic yeast*

Fifty six thermophilic yeast strains were isolated from seventy soil samples. Screening for bioethanol production revealed forty four strains with positive results as shown in Table 1 with strain IHZ-26 showing maximum bioethanol production i.e. 3.07 ± 0.05 g/L. This strain was further confirmed as yeast by observing budding formation in a range of 1.5 to 3 μm size using electron micrograph and therefore selected for further studies (Figure 1).

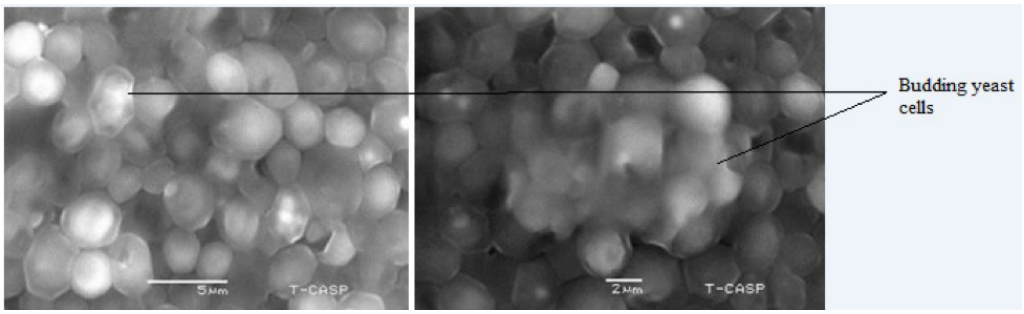

Fig. 1. Scanning electron micrograph (50X) of yeast strain IHZ-26 showing buds of yeast cells.

Table 1. Screening of yeast strains isolated from soil samples for sugar conversion ability to ethanol. The ± sign represents standard deviation between three determinants which differ significantly at $P \leq 0.05$.

Yeast Strains	Ethanol Production (g/L)	Yeast Strains	Ethanol Production (g/L)
IHZ-1	0.6±0.10	IHZ-29	0.77±0.09
IHZ-2	1.2±0.04	IHZ-30	-
IHZ-3	1.3±0.01	IHZ-31	0.98±0.29
IHZ-4	1.7±0.01	IHZ-32	0.42±0.23
IHZ-5	0.6±0.09	IHZ-33	-
IHZ-6	0.2±0.04	IHZ-34	0.97±0.17
IHZ-7	-	IHZ-35	0.28±0.21
IHZ-8	0.16±0.01	IHZ-36	-
IHZ-9	0.69±0.01	IHZ-37	1.22±0.19
IHZ-10	0.21±0.05	IHZ-38	1.73±0.06
IHZ-11	0.75±0.11	IHZ-39	-
IHZ-12	0.98±0.13	IHZ-40	1.78±0.13
IHZ-13	-	IHZ-41	-
IHZ-14	0.78±0.23	IHZ-42	1.96±0.11
IHZ-15	0.3±0.06	IHZ-43	-
IHZ-16	1±0.10	IHZ-44	1.56±0.05
IHZ-17	1.21±0.18	IHZ-45	1.23±0.01
IHZ-18	-	IHZ-46	0.11±0.01
IHZ-19	1.65±0.19	IHZ-47	0.9±0.09
IHZ-20	1.01±0.21	IHZ-48	0.6±0.21
IHZ-21	0.23±0.02	IHZ-49	1.7±0.11
IHZ-22	-	IHZ-50	2.1±0.30
IHZ-23	1.68±0.05	IHZ-51	1.9±0.05
IHZ-24	1.48±0.03	IHZ-52	1.3±0.15
IHZ-25	-	IHZ-53	0.6±0.03
IHZ-26	3.07±0.15	IHZ-54	0.2±0.08
IHZ-27	-	IHZ-55	1.86±0.10
IHZ-28	0.87±0.10	IHZ-56	1.14±0.02

Soil from vicinities of sugar industries was collected for isolation because chances of isolating microorganisms having ethanol production ability is comparatively greater in those areas due to the availability of high sugar content in the soil (Buddiwong *et al.*, 2014). The microbes isolated were mainly different species of yeast. Colony characteristics of isolated yeast strains were observed

which showed considerable diversity in the isolates. Thancharoen, (2016) also reported the diversity of yeast strains isolated from soil, based on their colony morphology.

3.2 Molecular identification

ITS region of yeast strain IHZ-26 was amplified by PCR and agarose gel electrophoresis was carried out for product analysis (Figure 2). The amplified ITS region of IHZ-26 was 618 bp. BLAST of the gene sequence indicated that IHZ-26 have 100% identity with *Wickerhamomyces anomalus* (KJ451674.1). Then, multiple alignment of query sequence with 15 other known sequences of *Wickerhamomyces anomalus* and *Pichia* sp. obtained after BLAST expressed as dendrogram (Figure 3) confirmed isolate IHZ-26 as a new strain of *Wickerhamomyces anomalus* (KT883963). Ramos et al (2017) also identified fungal strain using ITS region sequence with subsequent PCR amplification, BLAST application and Homology analysis.

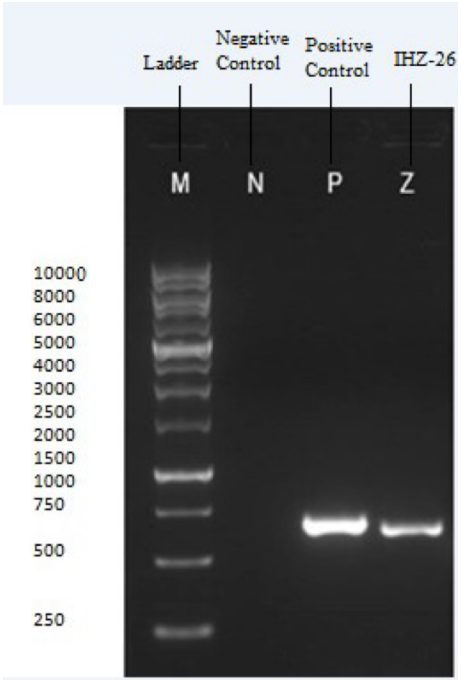

Fig. 2. **Gel electrophoresis profile of PCR product** (**M**: Ladder (AMPIGENE® DNA Ladder 250-10,000 bp); **N**: No template control; **P**: Positive control (DNA extracted from *Flammulina velutipes* is used as tempelate) and *Z*: Sample (PCR product of ITS region).

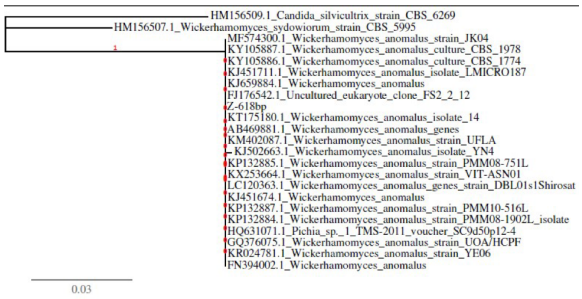

Fig. 3. Phylogenetic tree of IHZ-26 (isolate) along with related species obtained after BLASTn. The vast majority of species shown in dendogram are closely related to each other as indicated by branch length value (1).

3.3 Ethanol tolerance

For checking the ethanol tolerance potential of IHZ-26, the strain was cultured using different concentration of ethanol (5, 10, 15, 20 and 25% (v/v)) along with control having no ethanol. IHZ-26 was observed to be 100% tolerant up to 10% (v/v) ethanol concentration. A slight decrease in tolerance (98±0.02%) was observed at 15% (v/v) ethanol concentration which was further dropped to 80±0.06% at 20% (v/v) ethanol concentration. Further increase in the ethanol concentration up to 25% (v/v) showed drastic decrease (21±0.01%) in ethanol tolerance. Ethanol tolerance of IHZ-26 was estimated as 80% at 20% (v/v) ethanol concentration which is in accordance with findings of Mukherjee *et al*., 2017. The exact mechanism and physiological background for ethanol tolerance is still ambiguous.

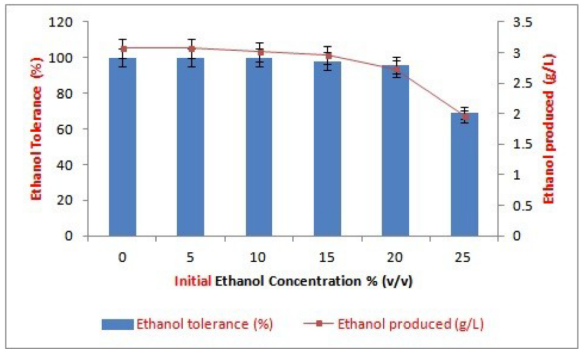

Fig. 4. Ethanol-tolerance assessment and effect of different ethanol concentration on sugar conversion ability of *Wickerhamomyces anomalus* (IHZ-26) to ethanol. Error bars represent standard deviation (±SD) between three determinants which differ significantly at $P \leq 0.05$.

On the other hand, ethanol production ability was decreased negligibly to 3.01±0.03 g/L, 2.96±0.02 g/L and slightly to 2.73±0.02 g/L at ethanol concentration of 10, 15 and 20% (v/v), respectively, as compared to control (3.07±0.01 g/L) (Figure 4). However, a radical decrease in ethanol production (1.96±0.09 g/L) was observed at 25% (v/v) ethanol concentration. This ability of ethanol production at high alcohol concentration might be attributed to some characteristics of cell wall that maintain its integrity and protects protein inside cell from denaturation, since it is linked directly to the surrounding medium (Desai *et al*., 2013; Ding *et al*., 2009).

3.4 Thermo-tolerance

Effect of different temperatures (45, 50, 55, and 60 ºC) on the growth and ethanol production ability of *Wickerhamomyces anomalus* IHZ-26 was assessed. It showed stable growth up to 50 ºC. Beyond that temperature, a considerable decrease in growth was observed; which was maximum at 60 ºC (19±0.05%) as shown in Figure 5. However, a gradual increase in ethanol production was noted from 45 ºC (3.07±0.12 g/L) to 50 ºC (3.91±0.06 g/L). A dramatic decline in ethanol production was observed at 55 ºC (1.87±0.02 g/L) and 60 ºC (0.12±0.01 g/L), concomitantly with decrease in yeast growth pattern. High temperature may have stressful effects on microbes due to Heat Shock Proteins (HSPs) synthesis which causes the disturbance in activity of ribosomes. Moreover, denaturation of enzymes at elevated temperature may have caused inactivation in yeast metabolism (Wu *et al*., 2016).

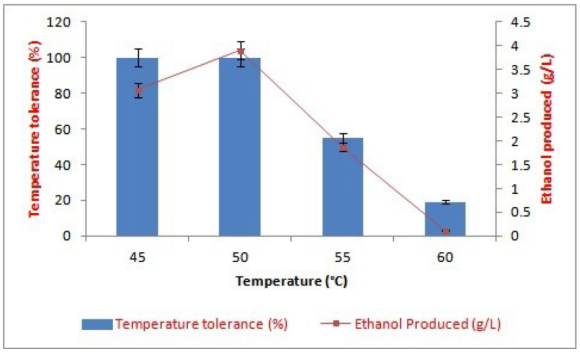

Fig. 5. Thermo-tolerance analysis and effect of different temperatures on sugar conversion ability of *Wickerhamomyces anomalus* (IHZ-26) to ethanol. Error bars represent standard deviation (±SD) between three determinants, which differ significantly at $P \leq 0.05$.

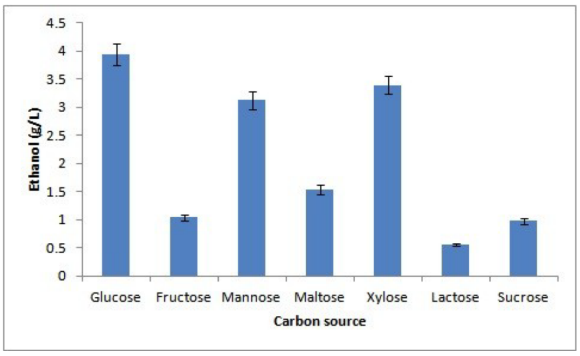

Fig. 6. Multiple sugars fermentation potential of *Wickerhamomyces anomalus* (IHZ-26). Error bars represent standard deviation (± SD) between three determinants which differ significantly at $P \leq 0.05$.

The results of current study are not in accordance with the findings of Cappelli *et al*., (2014) who reported 40 ºC as suitable temperature for *Wickerhamomyces anomalus* growth and ethanol production which makes the present study strain more potent and efficient.

3.5 Multiple sugars utilization

The *Wickerhamomyces anomalus* IHZ-26 was analyzed for conversion of different carbohydrates using glucose, fructose, mannose, maltose, xylose and lactose into ethanol. Among these, 3.94±0.06 g/L is the maximum production of ethanol that was observed using glucose as carbon source. Interestingly, appreciable conversion of xylose into ethanol i.e. 3.40±0.012 g/L was also observed. Ethanol production against fructose, mannose, maltose, lactose and sucrose was observed as 1.04±0.06 g/L, 3.13±0.01 g/L, 1.54±0.04 g/L, 0.56±0.01 g/L and 0.98±0.02 g/L, respectively (Figure 6). Disaccharides utilization may be facilitated by their hydrolysis through different enzymes such as invertase and α-amylase produced by yeast. Results of this study are in accordance with Zhang *et al*., (2014) and Kricka *et al*., (2014) who reported fermentation of various simple and complex sugars using *Wickerhamomyces anomalus*.

Conclusions

The *Wickerhamomyces anomalus* (IHZ-26) strain eliminated the need of two different yeast species for fermentation of pentoses and hexoses as it can ferment

both, simultaneously, unlike those reported previously. Moreover, high temperature tolerance results in elimination of cooling process required to maintain the mesophilic temperature for yeast growth in industries situated in the regions with elevated temperatures. This furthers adds to concomitant recovery of ethanol thus simplifying the downstream processing. Ethanol tolerance ability of the strain will reduce the yeast cells inhibition due to high ethanol concentration, thus more efficient yields of ethanol may be achieved. Based upon above mentioned properties, the use of said strain may lead to the development of more productive thus economical industrial process for bioethanol production. However, application of this strain at semi pilot and pilot scale still needs to be assessed before employing this strain in industries.

Acknowledgements

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