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Lignocellulosic bioethanol production employing newly isolated inhibitor and thermotolerant *Saccharomyces cerevisiae* DBTIOC S24 strain in SSF and SHF

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Abstract

Bioethanol is a renewable alternative to fossil fuels which facilitate energy security and reduce greenhouse-gas emissions. High gravity fermentation employing thermo and inhibitor tolerant strain is a promising technology to reduce fermentation time as well as cost. The present study investigates lignocellulosic ethanol production using inhibitor and thermotolerant *S. cerevisiae* DBTIOC S24 from non-detoxified and unsterilized rice straw hydrolysate. Efficient ethanol production was observed at wide range of pH (3 - 7) and temperatures (25 - 42 °C) using *S. cerevisiae* isolate. In presence of lignocellulosic derived inhibitors, maximum 75.33 g L^{-1} (85.56 %) and 73.30 g L^{-1} (79.93 %) ethanol was produced at 30 °C and 42 °C, respectively. During

fermentation, pH plays an important role to overcome the synergistic effect of inhibitors. More than 80.65 % and 73.5 % ethanol yield was achieved employing this isolate with high solid loading (20 %) and 20 FPU g^{-1} of solid loading via simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF), respectively. While, 91 % ethanol yield obtained during fermentation using of rice enzymatic hydrolysate. These values are comparable to the best results reported. Therefore, this isolate has great potential due to its inhibitors and thermo tolerant characteristic for lignocellulosic ethanol production at industrial scale with lower process time and cost.

Keywords: *Saccharomyces cerevisiae*; Lignocellulosic ethanol; Simultaneous saccharification and fermentation (SSF); Separate hydrolysis and fermentation (SHF); Thermo-tolerant; Inhibitor tolerant

1. Introduction

The global energy demand inexorably increased in last couple of years and projected to further increase more than 37% by 2040.[1] Presently, the global energy demand is fulfilled mainly by fossil fuel, which is a major concern at present due to its limited availability and high negative impact on environment.[2] Biofuel especially bioethanol looks promising as an alternative for fossil fuel. At present, most of supply comes first generation, which is limited by production cost, compete with land, water, food and fiber.[3] An alternative, second generation bioethanol from lignocellulosic biomass is fascinating due to its surplus availability worldwide. Among the lignocellulosic biomass, rice straw is the most abundant in world.[4] In addition, rice straw has a significant content of silica, therefore not suitable for pulp & paper industry and animal fodder.[5]

Hence, rice straw could be considered as potential agriculture residue for lignocellulosic ethanol production.

Lignocellulosic biomass is recalcitrant complex structure, which requires a pretreatment before enzymatic hydrolysis to make accessible cellulose for enzymatic hydrolysis. Among the available pretreatment technologies, dilute acid pretreatment considered one of the most efficient, cost effective and closest to commercialization.[6] However, the inherent disadvantage of this technology is production of inhibitors which reduces enzyme activity as well as fermentation efficiency.[7,8] The various detoxification strategies for hydrolysate or slurries include alkali or sulfite treatment, liquid-liquid extraction, ion exchange and treatment with enzyme but these detoxification methods were costly and results in loss of sugars, hence not considered suitable for an economically viable technology.[8,9]

Fermentation process is exothermic in nature and causes arise in temperature during industrial scale fermentation because these are operated adiabatically due to much lower surface to volume ratio compared to laboratory fermentor.[10] Therefore, application of thermotolerant strain for ethanol production is highly warranted with its potential to reduce cooling cost along with cessation during ethanol fermentation due to overheating problem.[11] Among ethanologenic organisms, *S. cerevisiae* considered as an industrial strain for bioethanol production however, it is susceptible for the lignocellulosic inhibitors and other stress conditions viz. pH, temperature, substrate concentration etc.[8] Various studies have shown that ethanol yield and productivity were significantly reduced below 35 °C using thermo tolerant strains and vice-versa. Significant effort have been made for developing thermo-tolerant and inhibitor tolerant trait in ethanologenic microorganism using protoplast fusion, genetic engineering, genome shuffling, mutation and

evolutionary engineering.[12] However, most of the studies addressed for tolerance of one or two specific stress, thus not useful for combined tolerance for multiple stresses including temperature and inhibitors. Therefore, employing natural thermo and inhibitor tolerant ethanologenic yeast with tolerance for multiple stresses is realistic approach for viable economics of second generation biofuel production.[13]

Considering above facts, present study focuses on isolation of thermo and inhibitor tolerant yeast strain from distillery spent wash as this habitat is acidic in nature, high organic material loading, exposed directly to sunlight leading to increase in temperature, low dissolved oxygen and high solid contents.[14,15] The isolated strain was thoroughly examined for synergistic effect of inhibitors on ethanol fermentation at high temperature. Further, isolate was evaluated for ethanol fermentation via separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) using unsterilized and non-detoxified rice straw hydrolysate at elevated temperature. To the best of our knowledge, this is the first report using non-detoxified and unsterilized rice straw hydrolysate for ethanol production employing inhibitor and thermo tolerant *S. cerevisiae* at 42 °C in bioreactor.

2. Materials and Methods

2.1 Sample collection, media and chemicals

To isolate ethanol fermenting yeasts, eight soil and spent wash samples were collected in sterile bottles from various sites of three different distillery and sugar mill from National Capital Region, India. YPD broth (yeast extract 10 g L^{-1}, peptone 20 g L^{-1}, glucose 20 g L^{-1}) was used for isolation of yeast. For solid medium, 2 % (w/v) agar was added into broth. Two other media

were used for screening process. One was pre-culture medium (PCM) consisted of yeast extract 10 g L^{-1}, peptone 20 g L^{-1}, glucose 50 g L^{-1} and ethanol 80 g L^{-1}. Second yeast fermentation medium (YFM) was used to evaluate fermentation efficiency of yeast isolates. YFM media was consisted of yeast extract 2.5 g L^{-1}, yeast nitrogen base 1.7 g L^{-1}, ammonium sulfate 5.0 g L^{-1} , magnesium sulfate 6.0 g L^{-1} and glucose 60 - 180 g L^{-1}. All the media were adjusted to pH 5.0±0.2 with 1 M HCl and 1 M KOH, autoclaved at 121 °C and15 Lb pressure for 20 min.

2.2 Isolation, screening and selection of yeast isolates

Soil/ spent wash sample (10 g) was dispersed in 100 ml saline (0.85 %) and mix thoroughly. After appropriate dilution, 100 µl samples was spread on YPD agar plate containing 60 g L^{-1} ethanol to enrich only ethanol tolerating strains and 0.05 g L^{-1} streptomycin to prevent bacterial growth, and incubated at 30 °C for 72 h. Twenty six yeast colonies were appeared through the soil serial dilution plate method. All 26 yeast colonies were subjected to further screening and selection using PCM broth and agar plates. Selected yeast colonies were evaluated for their fermentation ability using YFM broth amended with 180 g L^{-1} glucose at 42 °C at 180 rpm for 48 h. The seed culture for fermentation was prepared by growing yeast in 2 liter of YPD media for 16 hours (mid-log phase culture) and centrifuged at 5000 rpm for 10 min. The cell pellet was dissolved in 0.85 % saline. The inoculum was transferred to screening media, to give initial DCW of 1.0 g L^{-1}.

2.3 Identification and characterization

Based on the fermentation efficiency, a yeast strain (DBTIOC S24) was selected for further studies and characterized by sequencing. Yeast DNA was isolated using DNeasy blood & tissue

kit (Qiagen). The internal transcribed spacer region (ITS) 5.8S rDNA and the adjacent ITS1 and ITS2 regions were amplified by polymerase chain reaction (PCR) using the primer pairs ITS1 5′-TCCGTAGGTGAACCTGCG-3′ and ITS4 5′-TCCTCCGCTTATTGATATGC-3′.[16] PCR amplification was performed in 50 µl reaction mixtures containing approximately 20 ng of genomic DNA template, 1× PCR buffer with 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 µM of each primer and 1 U Taq polymerase. PCR cycling conditions were 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 45 s, followed by an extension step of 72 °C for 10 min. The amplified DNA was purified using *Qiaquick* PCR Purification Kit (Qiagen) and sequenced. The sequences were BLASTn against NCBI data base. The 99% and higher sequence match were considered for species identification. The phylogenetic dendorgram was prepared using Molecular Evolutionary Genetics Analysis (MEGA) software version 5.0.[17] Substrate utilization profile of the yeast isolate was studied using a HiCarbo™ Kit (HiMedia) following manufacturer's instructions.

2.4 Ethanol fermentation at different pH, temperatures and substrate concentrations

To study the influence of pH on fermentation efficiency, the initial pH of the YFM supplemented with 180 g L^{-1} glucose was adjusted to the desired value (3-7) using sterile HCl and KOH solutions. The flasks were inoculated and incubated at 30 °C and 180 rpm for 72 h. Similarly, temperature variation experiment was setup at pH 5.0 and incubates the flasks at 25 °C, 30 °C, 37 °C and 42 °C for 48 h. At different time intervals, samples were withdrawn and analyzed for cell growth, residual glucose and ethanol content. Similar to above, effect of initial glucose concentration (60, 120 and 180 g L^{-1}) on fermentation efficiency of selected strain at 30 °C and 42 °C was also evaluated.

2.5 Synergistic effect of ligno-cellulosic inhibitory compounds on ethanol fermentation

In order to study synergetic effect of inhibitors on fermentation efficiency of isolated yeast, four inhibitors were used viz. acetic acid (0 - 4.5 g L^{-1}), formic acid (0 - 1.0 g L^{-1}), 5-HMF (0 - 3.0 g L^{-1}) and Furfural (0 - 2.0 g L^{-1}) in 15 different combinations with and without pH adjustment (5.0±0.2) by using HCl or KOH (Table 1). For fermentation experiments, 250 mL Erlenmeyer flask with 100 ml YFM was inoculated with overnight grown culture (initial cell concentration 1.0 g L^{-1}). After 48 h, samples were withdrawn and analyzed for cell growth, residual glucose and ethanol content.

2.6 Ethanol fermentation in absence and presence of inhibitor cocktails

To mimic actual inhibitor concentration present in hydrolysates, batch fermentation was performed at 30 °C, 37 °C and 42 °C in 7.5 liter NBS Bioflow 115 bioreactor using 3 L YFM (180 g L^{-1} glucose) amended with inhibitors (acetic acid 2.5 g L^{-1}; formic acid 0.25 g L^{-1}; 5-HMF 0.75 g L^{-1} and furfural 0.6 g L^{-1}) at pH 5±0.2 and 200 rpm. A parallel bioreactor was also run without any inhibitor as a control. Samples were withdrawn at different time intervals and analyzed for cell growth, residual glucose and ethanol content. The fermentation was started by inoculating yeast cell at initial DCW of 1.0 g L^{-1}.

2.7 Ethanol fermentation using hydrolysate via SHF and SSF

Dilute acid pretreatment of rice straw was performed in screw type pilot scale continuous pretreatment reactor using 0.3% sulphuric acid at 162 °C for 15 min as described by Saini et al.[18] and resulted pretreated slurry contains 28.4% of total solid. The Water insoluble solid (WIS) of slurry was consisting of 51.4% glucan, 3.9% xylan and 28.9% lignin. Dilute acid pretreated rice

straw slurry was enzymatic hydrolyzed without any detoxification, washing, sterilization and filtration step at 20 % (w/w) solid loading [water insoluble solid (WIS) 15.2 %, glucose 8 g L^{-1}, xylose 32 g L^{-1}) using 20 FPU *SacchariSEB C6L* (Advanced Enzymes, India) per g of solid loading in NBS Bioflow 115 bioreactor (3 L working volume, pitch blade impeller) at 50 °C. The 20 FPU of enzyme *SacchariSEB C6L* contains 48.09 U of β-glucosidase, 160.00 U of Endoglucanase and 7.34 mg protein.[19] Resulted enzymatic hydrolysate was used in fermentation experiments employing isolated yeast in bioreactor containing 3 L hydrolysate amended with yeast extract (2.5 g L^{-1}), yeast nitrogen base (1.7 g L^{-1}), ammonium sulphate (5.0 g L^{-1}) and magnesium sulphate (6.0 g L^{-1}). Yeast cells were inoculated with an initial DCW of 2.0 g L^{-1} and incubated at 30 °C and 42 °C for 48 h at 200 rpm. Two parallel bioreactors were also run using YFM amended with 80 g L^{-1} glucose employing yeast isolate at 30 °C and 42 °C as a control. Samples were withdrawn at different time intervals and analyzed for glucose and ethanol concentration.

Similarly, SSF experiment was also executed in NBS Bioflow 115 bioreactor (2 L working volume, pitch blade impeller) at 42 °C, 20 % (w/w) solid loading and 20 FPU *SacchariSEB C6L* per g of solid loading. Pre-saccharification was performed at 50 °C for initial 3 h followed by SSF for 45 h. Another set of SSF was carried with 25 % solid loading without pre-saccharification step. Rest experimental conditions were same as previous. Samples were withdrawn at different time intervals and analyzed for glucose and ethanol concentration.

2.8 Analytical techniques

To determine the sugar, inhibitors and ethanol concentration in media, samples were collected and centrifuged at 5000 rpm for 10 min. Supernatant was kept at -20 °C till analysis.

Quantitative analysis of sugars and inhibitors (5-HMF, Furfural, Acetic Acid were conducted by HPLC equipped with an Aminex HPX-87H column 300 mm × 7.8 mm ID (*BioRad Labs*). Sugars and acetic acid were quantified by refractive index detector while, 5-HMF and Furfural were quantified through PDA detector (UV/Vis detector at 254 nm). The mobile phase was 50 mM H_2SO_4 with an elution flow rate of 0.6 mL min^{-1}. Column and detector temperatures were 50 °C and 30 °C, respectively.

Ethanol estimation was done by Gas Chromatograph (Clarus 680 *PerkinElmer*) fitted with Elite-5 MS column (30 m × 0.32 mm × 0.25 μm) using the following temperature program: initial 60 °C (held for 3 min) to 150 °C at the rate of 10 °C min^{-1}. Injector and detector temperatures were 150 °C and 250 °C, respectively. Helium was taken as a carrier gas at 2 mL min^{-1} flow rate. The dry cell weight (DCW) was measured by converting cell absorbance (λ_{600}) using 5 point calibration standard.

2.9 Statistical analysis and equations

All the studies were conducted in triplicates and the results are presented as means of the replicates along with standard deviation (represented as error bars). Data were analyzed by using one way ANOVA.

Equations 1 - 4 were applied for synthetic media and SHF process whereas, Equation 5 was applied to calculate ethanol yield in the SSF processes.

$$Ethanol\ productivity\ Q\ (g\ L^{-1}\ h^{-1}) = \frac{E_t - E_o}{t} \quad \text{..... (1)}$$

$$Ethanol\ specific\ productivity\ q\ (g\ g^{-1}\ h^{-1}) = \frac{Q_t}{X_t}$$

…….…… (2)

$$Etahnol\ yield\ (g\ g^{-1}) = \frac{E_t}{G_t}$$

……..…… (3)

$$Etahnol\ yield\ (\%) = \frac{E_t}{(G_t \times 0.51)} \times 100$$

……...…….……..… (4)

$$Ethanol\ yield\ (\%) = \frac{[E]}{[(TS - WSS) \times f \times 1.111 \times 0.51] + (S \times 0.51)} \times 100$$

……...…….……..… (5)

Where, E indicates total ethanol produced during fermentation (g L^{-1}), X_t indicates biomass (g L^{-1}) after time *t*, G_t indicates consumed glucose (g L^{-1}) after time *t, TS* is the total solid biomass (g L^{-1}), *WSS* indicates water soluble solid, '*f*' is cellulose fraction of dry biomass, *S* is glucose content in biomass before pre-saccharification, 0.51 is the conversion factor for glucose to ethanol based on stoichiometric biochemistry of yeast and 1.111 is the conversion factor for cellulose to equivalent glucose.

3. Results and discussion

3.1 Screening, isolation and identification of inhibitors and thermo tolerant yeast

In the present study, 26 morphologically yeast colonies were isolated from eight samples on YPD agar medium supplemented with streptomycin and ethanol. In order to obtain ethanol tolerant yeast strains, enrichment isolation were carried out in PCM agar and broth medium at 30 °C. Five yeast strains among all grown on PCM agar and broth medium. These strains were

compared on the basis of their fermentation efficiency along with reference strain *S. cerevisiae* NRRL2034 at 42 °C for 48 h. Comparative ethanol production results are shown in Fig. 1. Remarkably, DBT-IOC S24 strain performed much better in ethanol production (69.47 g L^{-1}) and yield (81.16 %) than other isolates and reference strain *S. cerevisiae* NRRL2034. No strains were capable to grow and ferment sugar at 45 °C (data not shown). Distillery/sugar mill spent wash is a good habitat for efficient inhibitor and thermotolerant natural strains.

To identify DBTIOC S24 isolate, highly variable region of partial 18S rRNA, ITS1, 5.8S rRNA, ITS2 and partial 28S rRNA gene was amplified, sequenced, matched against NCBI data base (http://blast.ncbi.nlm.nih.gov) and results were used to construct phylogenetic tree (supplementary Fig. A.1). As per phylogenetic tree, yeast isolate DBTIOC S24 is closely related (> 99% similarity) to *S. cerevisiae*. Hence, it was identified and designated as *S. cerevisiae* DBTIOC S24 (NCBI GenBank Accession Number: KT375337). In view of screening results, DBTIOC S24 strain was selected for further evaluation and characterization. The biochemical properties for substrate utilization were positive for fructose, dextrose, galactose, rafinose, trehalose, mannose, inulin, salicin, melezitose and esculin (Table 2).

3.2 Fermentation performance of DBTIOC S24 at various temperatures, pH and initial glucose concentration

Tolerance for high temperatures, inhibitors, pH, ethanol concentration and high sugar concentrations are the main barriers for any yeast during fermentation. Thus, the characterization of yeast tolerance to these stresses is essential for efficient lignocellulosic ethanol fermentation.[20,21] As result shown in Fig. 2A, *S. cerevisiae* DBTIOC S24 isolate consumed complete glucose and produce maximum 83.12 g L^{-1} ethanol at 30 °C. Higher glucose

consumption rate was observed in initial 24 h in all the individual temperature sets. At 42 °C, ethanol yield was more than 84.94 % but glucose consumption rate and ethanol productivity decreased, which could be due to combined inhibitory effects of produced ethanol and higher temperature. Reduced growth and lower ethanol production at high temperature during fermentation is reported elsewhere.[13,22] At 25 °C, Minimum 77 % ethanol yield was observed after 24 h while, 84 – 90 % ethanol yield were recorded in rest of temperature sets, is noteworthy.

Further, to evaluate the fermentation efficiency of yeast at various pH, DBTIOC S24 isolate was inoculated in YFM (180 g L^{-1} glucose) with different initial pH (3 - 7) and incubated at 30 °C. Fig. 2B represents the glucose and ethanol content after 24, 48 and 72 h. Complete glucose was consumed within 24 h in broth medium in initial pH 5 - 7 range while, at pH 3 - 4, almost complete glucose consumption was possible only after 48 h. The maximum production of ethanol (85.17 g L^{-1}) was achieved within 24 h when initial pH was 6. The ethanol yield was observed in rage of 81 – 94 % in all pH variation sets with maximum at pH 6. The highest ethanol productivity 3.55 g L^{-1} h^{-1} was obtained at pH 6 followed by pH 5 (3.23 g L^{-1} h^{-1}). Therefore, DBTIOC S24 shows activity in much wider range of pH. Optimum pH for maximum ethanol fermentation was reported from pH 4.5 - 5.5 using *S. cerevisiae*.[20,23] Principally, it depends upon ΔpH i.e. pH difference of inside and outside pH of yeast cell.[24]

The cost effective and low negative impact on environment, lignocellulosic ethanol production can be a lucrative option using high gravity fermentation. Therefore, it is worthwhile to examine the effect of different glucose concentrations (60, 120, 180 g L^{-1}) on fermentation efficiency of DBTIOC S24 isolate. As shown in Fig. 2C, complete glucose was consumed in all the sets with

in 24 h at both the temperatures except the one with 180 g L^{-1} initial sugar concentration at 42 °C. At 30 °C, 78.53 g L^{-1}, 54.08 g L^{-1} and 27.89 g L^{-1} ethanol were produced after 24 h with initial glucose concentration 180 g L^{-1}, 120 g L^{-1} and 60 g L^{-1}, respectively. Remarkably, ethanol yield were recorded in the range of 86 – 93 % and 82 – 86 % at 30 °C and 42 °C, respectively. Considering the above observations, DBTIOC S24 isolate can be used for efficient ethanol fermentation at wide range of temperature and pH using high gravity fermentation with cost effective down streaming.

3.3 Synergistic effect of lignocellulosic inhibitors cocktails on ethanol fermentation

Ethanol fermentation in the presence of inhibitors which can cause the slowing or cessation of microbial cell growth, reduced ethanol productivity and low ethanol yield was a challenge. In order to study dose dependent response against the key lignocellulosic inhibitors cocktails, the effect of acetic acid (0 - 4.5 g L^{-1}), formic acid (0 - 1.0 g L^{-1}), 5-HMF (0 - 3.0 g L^{-1}) and Furfural (0 - 2.0 g L^{-1}) on fermentation efficiency of DBTIOC S24 isolate was investigated. However, by adopting acid pretreatment or steam explosion and employing severe conditions, the inhibitors concentration was much lower than the concentration range evaluated in our study.[7,25] As indicated in Table 1, fifteen different combinations of inhibitors cocktails with and without pH adjustment (5.0±0.2) were inoculated and incubated for 46 h. Without adjusting initial pH, maximum 2.17 g L^{-1} biomass and 76.81 g L^{-1} ethanol were produced in absence of inhibitors while, ethanol concentration and productivity decreased in presence of inhibitors cocktails. Ethanol concentration and productivity drastically decrease with increase in weak acids concentration to above 0.66 g L^{-1}. The effects of weak acids are strongly pH dependent. At pH value below the pKa-value of the acid, the undissociated form of weak acids predominates.

Undissociated acids enter inside the cell through passive diffusion and get dissociated due to higher internal pH. Hydrogen ions are pumped out through an ATP coupled reaction and taken potassium ion to maintain ionic stasis. Although low levels of acids activate the glycolytic rate by stimulating ATP production, higher levels become inhibitory due to the acidification of the cytosol after depletion of the available ATP, resulting inhibitory to several glycolytic enzymes.[24,26] The inhibitory effect of these compounds can be greatly overcome by adjusting the initial pH of medium to 5.0±0.2. After adjusting initial pH at 5.0, tolerance level of *S. cerevisiae* DBTIOC S24 isolate also increased. Nevertheless, maximum 78.89 g L^{-1} ethanol was produced in presence of inhibitors followed by in absence of inhibitors (77.04 g L^{-1}). At higher pH values, a reason for reduced inhibition due to the smaller ΔpH and less stress on cell because decreased intake concentration of undissociated acid.[24,27] Other than weak acids, furans (furfural and 5-HMF) also plays significant role to inhibit yeast growth and ethanol productivity. The inhibitory effect of furfural on growth and fermentation was enhanced by increasing the furfural concentration. These results agree with those obtained by Palmqvist et al.[28] using *S. cerevisiae* in the presence of furfural (4 g L^{-1}). Unlike the fufural, toxic effect of 5-HMF was not significant (up to 2 g L^{-1}) compared to furfural and acetic acid in cocktails. After adjusting the pH, toxic effect of inhibitors occur after certain concentration. Remarkably, inhibitors effect was much less as compared to the yeast strains reported in literature.[13] Bellido et al.[29] also observed that an increase in acetic acid and furfural concentration led to a reduction in sugar consumption rates and ethanol concentration with increasing concentration while 5-HMF did not exert a significant effect. Nevertheless, the DBTIOC S24 isolated shows much better tolerance to inhibitors cocktail and above findings also suggested the role of pH to overcome the inhibitors effect on ethanol

fermentation efficiency of yeast. This strain may serve as a potential candidate for economically viable lignocellulosic ethanol production at industrial scale.

3.4 Ethanol fermentation in presence of ligno-cellulosic inhibitors in bioreactor

To validate the versatility of *S. cerevisiae* DBTIOC S24 isolate, synthetic medium was amended with high sugar concentration and multiple inhibitors to create a high gravity multi-stress fermentation environment in bioreactor. Fig. 3 indicates dry cell weight (DCW), glucose concentration, ethanol concentration and ethanol yield at different time and temperatures. From the Fig. 3, it clearly depicted that 37 °C was the optimum temperature for growth. Maximum 5.54 g L^{-1} yeast grown with in 20 h in control followed by 5.52 g L^{-1} yeast grown with in 42 h in presence of inhibitors at 37 °C. This could be due to increased lag phase of yeast isolate in inhibitor amended medium. In terms of ethanol fermentation, maximum 91.05 g L^{-1} ethanol (22 h) was produced at 30 °C followed by 81.07 g L^{-1} at 37 °C (20 h) and 80.68 g L^{-1} at 42 °C (42 h) in control conditions. The ethanol yields were varied from 88 – 96 % (0.45 g g^{-1} - 0.49 g g^{-1}). Sree et al.[30] reported 0.48 g g^{-1} (at 30 °C) and 0.36 g g^{-1} (40 °C) ethanol yield employing *S. cerevisiae*. Ortiz-Muniz et al.[31] reported maximum 0.41 g g^{-1} ethanol yield of *S. cerevisiae* ITV-01 at 30 °C. In presence of inhibitors, maximum 78.02 g L^{-1} ethanol (82 %) was produced with in 42 h at 37 °C followed by 75.33 g L^{-1} at 30 °C (28 h) and 73.30 g L^{-1} ethanol at 42 °C (64 h). Since, inhibitors were incapable to significantly affect the fermentation efficiency of *S. cerevisiae* DBTIOC S24 at higher temperature, therefore it may be useful to save cooling cost and reduce contamination chances at industrial scale.

3.5 Ethanol fermentation via SHF

316 In this section, *S. cerevisiae* DBTIOC S24 isolate was evaluated for efficient ethanol
317 fermentation using lignocellulosic hydrolysate because actual hydrolysate differs from the
318 synthetic cocktails due to unidentified inhibitors which shows synergistic effect on yeast
319 fermentation efficiency.[9] Fermentation performance of DBTIOC S24 isolate was evaluated via
320 SHF at 30 °C and 42 °C using pretreated rice straw on account of exothermic nature of
321 fermentation process and fair chances to increase in temperature in the industrial scale bioreactor
322 due to negligible heat loss to environment.

323 SHF process was performed using dilute acid pretreated rice straw at 20% total solid loading
324 using *SaccharISEB C6L* enzyme which resulted into a rice straw hydrolysate slurry containing
325 glucose, 80.5 g L^{-1}; xylose, 30.2 g L^{-1}; acetic acid, 1.86 g L^{-1}; 5-HMF, 0.52 g L^{-1}; furfural, 0.21 g
326 L^{-1} . The enzymatically hydrolyzed slurry was used as such for fermentation at 30 °C and 42 °C.
327 Fig. 4A illustrates glucose and ethanol concentration along with ethanol yield at different time
328 interval. At 30 °C, maximum glucose gets exhausted in initial 6 h and produced maximum 35.30
329 g L^{-1} ethanol which is corresponding to 91% ethanol fermentation efficiency while, 73.5 %
330 ethanol yield was calculated after considering initial glucose and cellulose content of pretreated
331 slurry. The furfural was completely metabolized in initial 3 h whereas, 5-HMF conversion
332 comparatively slower and remain 0.26 g L^{-1} after 6 h. During the fermentation process, two main
333 inhibitors compound relevant to lignocellulosic biomass i.e., Furfural and 5-HMF are
334 metabolized by yeast to their corresponding less inhibitory alcohol form.[21,32] At 42 °C, maximum
335 33.93 g L^{-1} ethanol was produced within 29 hours with 83.73 % fermentation efficiency achieved
336 considering initial glucose in pretreated slurry. While, considering both initial glucose and
337 cellulose content, 70.6 % efficiency was recorded. Ethanol productivity at 42 °C was calculated
338 as 1.17 g L^{-1} h^{-1} which was lower than ethanol productivity at 30 °C (5.88 g L^{-1} h^{-1}). This could

be due to combined adverse effect of ethanol and temperature on yeast physiology mainly because of changes in cell membrane permeability, transport system, damage to cell wall etc.[22] Similar to SHF at 30 °C, furfural was completely metabolized in initial 3 h and 5-HMF remained as 0.46 g L^{-1} after 21 h at 42 °C. In order to evaluate glucose consumption and fermentation efficiency of *S. cerevisiae* DBTIOC S24 in absence of inhibitors (as control), YFM with 80 g L^{-1} glucose was inoculated with yeast isolate (Fig. 4B). At 30 °C, almost complete glucose was consumed after 5 h with maximum 36.17 g L^{-1} ethanol content. At 42 °C, complete glucose consumption was taken 7 h with maximum 31.72 g L^{-1} ethanol content. Above observations indicates that DBTIOC S24 isolate has capabilities to efficiently ferment non-sterilized and non-detoxified actual hydrolysate at wide range of temperature (30 - 42 °C).

3.6 Ethanol fermentation via SSF

To evaluate fermentation efficiency of DBTIOC S24 isolate via SSF, dilute acid pretreated rice straw biomass was carried out in 5 L bioreactor using 20 FPU (*SacchariSEB C6L*) per g of solid biomass. SSF with 20 % solid loading was executed by initial 3 h pre-saccharification at 50 °C followed by 45 h at 42 °C. Initial pre-saccharification step allows enzyme to work at optimal temperature resulting in increased liquefaction, which allows biomass easy to ferment. Fig. 5A illustrates glucose and ethanol concentration along with ethanol yield during SSF. The ethanol productivity during the first 18 h of SSF was 1.72 g L^{-1} h^{-1} with ethanol concentration of 30.95 g L^{-1}. After 45 hours, maximum 38.22 g L^{-1} ethanol was obtained with 0.85 g L^{-1} h^{-1} productivity, which corresponds to an overall ethanol yield 80.65 %.

In order to increase the ethanol titer, another SSF experiment was performed at higher solid loading (25 %) under similar conditions except the elimination of pre-saccharification step (Fig.

5B). Maximum 49.45 g L^{-1} ethanol with 83.70 % yield was produced after 72 h. Ethanol concentration was much higher than threshold concentration of ethanol (40 g L^{-1}) for distillation, making process cost effective.[31] Maximum ethanol productivity was observed after 16 h (2.12 g L^{-1} h^{-1}) which, was subsequently decrease with increase in fermentation time. On the basis of above observations, there was no significant effect on ethanol production after elimination of pre-saccharification step. Saini et al.[18] reported that SSF without pre-saccharification led to more ethanol production even at a faster rate. Feasible high solid loading and elimination of pre-saccharification step employing thermotolerant yeast would be an added advantage to reduce fermentation time and enhancing ethanol titer along with productivity.[18] Jung et al.[34] reported 70.7 % ethanol yield of *S. cerevisiae* using rice straw biomass. The isolate used in the present study has better ethanol fermentation efficacy than the other reported strain viz. 56.3 % yield using *S. cerevisiae*[35] and 77.7 % yield using *Kluveromyces marxianus*[33] from lignocellulosic biomass. Various new commercial cellulotic enzymes preparation contains lytic polysaccharide monoxoygenases (LPMOs) and it has great influence on enzymatic hydrolysis by oxidative cleavage of crystalline cellulose/ hemicelluloses. LPMOs needs oxygen or electron donor for their activity. Lignin has been speculated to be the electron supplier for the activity of LPMOs.[36] In the present study, pretreated biomass contains 28.9 % lignin which is speculating to act as an electron donor for LPMOs without hampering the efficiency of SSF process. Above results indicate that thermo and inhibitor tolerance yeast isolate is a lucrative option for SSF process using whole dilute acid pretreated slurry without detoxification. SSF ethanol yield were higher (80.65 %) in comparison to SHF (73.5 %), similar results was also reported.[19] This could be due to several factors like no substrate feedback inhibition of enzyme, less inhibition of enzyme by

inhibitors as DBTIOCS24 yeast bio-detoxifies hydrolysate to lower concentration; and high temperature reduces the chance of contaminations.

4. Conclusion

A new thermo and inhibitor tolerant yeast strain was isolated and identified as *S. cerevisiae* DBTIOC S24. The yeast was active in wide range of temperature (25 - 42 °C) and pH (3 - 7) with high ethanol fermentation efficiency and productivity. The tolerance of this strain to high concentration of lignocellulosic inhibitors differentiates this from the similar thermo-tolerant strains reported in literature. Therefore, *S. cerevisiae* DBTIOC S24 shows high potential for the industrial scale fermentation via both SHF and SSF. To best of our knowledge, this is the first report employing a thermotolerant *S. cerevisiae* isolate to produce 49.45 g L^{-1} ethanol with 83.7 % yield at 42 °C using unsterilized and non-detoxified rice straw hydrolysate via SSF. In turn, above isolate has capability to produce lignocellulosic ethanol production with reduction of fermentation time and process cost.

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460 **Table 1. Effect of synthetic inhibitor cocktails at different concentrations on the**
461 **fermentation efficiency of *S. cerevisiae* DBTIOC S24 isolate at 30 °C after 46 h using 180 g**
462 **L^{-1} initial glucose**

Conditions	S. No.	Inhibitor cocktails (g/L)				Ethanol concentration (g/L)	Biomass (g/L)	Yield (g/g)	Productivity (g/L/h)	Specific productivity (g/g/h)
		Acetic acid	Formic acid	5-HMF	Furfural					
Without pH adjustment (3.0 - 4.0)	1.	0	0	0	0	76.81	2.17	0.430	1.670	0.77
	2.	0	0.33	1	1.32	74.30	1.82	0.436	1.615	0.89
	3.	0	0.66	2	0.66	36.69	1.40	0.367	0.798	0.57
	4.	0	1	3	2	2.24	0.58	0.086	0.049	0.08
	5.	1.5	0	2	2	4.85	0.65	0.147	0.105	0.16
	6.	1.5	0.33	3	0.66	1.42	0.49	0.053	0.031	0.06
	7.	1.5	0.66	0	1.32	1.83	0.57	0.071	0.040	0.07
	8.	1.5	1	1	0	0.98	0.53	0.038	0.021	0.04
	9.	3	0	1	0.66	2.30	0.63	0.083	0.050	0.08
	10.	3	0.33	0	2	1.31	0.57	0.044	0.029	0.05
	11.	3	0.66	3	0	9.56	0.55	0.349	0.208	0.38
	12.	3	1	2	1.32	3.25	0.51	0.113	0.071	0.14
	13.	4.5	0	3	1.32	1.93	0.51	0.076	0.042	0.08
	14.	4.5	0.66	1	2	1.23	0.55	0.041	0.027	0.05
	15.	4.5	1	0	0.66	0.64	0.54	0.025	0.014	0.03
With pH adjustment at 5.0±0.2	1.	0	0	0	0	77.04	2.05	0.453	1.675	0.82
	2.	0	0.33	1	1.32	76.72	1.83	0.440	1.668	0.91
	3.	0	0.66	2	0.66	72.69	1.66	0.436	1.580	0.95
	4.	0	1	3	2	47.85	1.47	0.374	1.040	0.71
	5.	1.5	0	2	2	72.45	1.79	0.427	1.575	0.88
	6.	1.5	0.33	3	0.66	67.00	1.79	0.396	1.456	0.81
	7.	1.5	0.66	0	1.32	76.71	2.04	0.430	1.668	0.82
	8.	1.5	1	1	0	78.89	2.06	0.440	1.715	0.83
	9.	3	0	1	0.66	77.47	2.09	0.431	1.684	0.81
	10.	3	0.33	0	2	73.10	2.08	0.409	1.589	0.76
	11.	3	0.66	3	0	41.24	0.83	0.423	0.896	1.08
	12.	3	1	2	1.32	50.77	1.33	0.419	1.104	0.83
	13.	4.5	0	3	1.32	8.56	0.71	0.205	0.186	0.26
	14.	4.5	0.66	1	2	68.28	1.82	0.410	1.484	0.81
	15.	4.5	1	0	0.66	73.89	2.29	0.418	1.606	0.70

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464

Table 2. Substrate utilization characteristics of *S. cerevisiae* DBTIOC S24 isolate

Substrate	Results*	Substrate	Results*	Substrate	Results*
Lactose	+	Inulin	++	Rhamnose	-
Xylose	-	Na-Gluconate	-	Cellobiose	+
Maltose	-	Glycerol	-	Melezitose	++
Fructose	++	Salicin	++	α-Methyl D-mannoside	-
Dextrose	++	Dulcitol	-	Xylitol	-
Galactose	++	Inositol	-	ortho-Nitrophenyl-β-galactoside	-
Rafinose	++	Sorbitol	+	Esculin	++
Trehalose	++	Manitol	+	D-Arabinose	-
Melibiose	-	Adonitol	-	Citrate	+
Sucrose	++	Arabitol	-	Malonate	-
L-Arabinose	-	Erythritol	-	Sorbose	-
Mannose	++	α-Methyl D-glucoside	-	Control (negative)	-

* (+) assimilation; (++) fermentation; (-) no growth

Figure captions

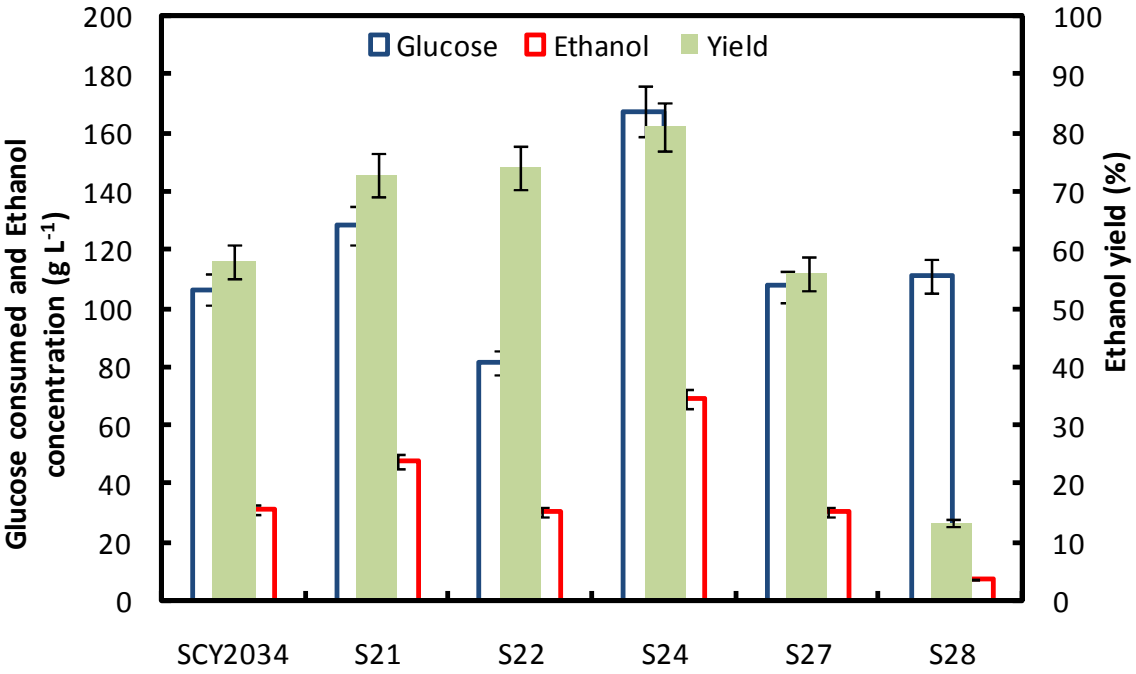
Fig. 1 Fermentation profile of yeast isolates in YFM Media (180 g L^{-1} initial glucose concentration, 42 °C, 180 rpm, pH 5.0±0.2, and 48 h) Reference strain SCY2034: *S. cerevisiae* NRRL2034

Fig. 2 Effect of temperature, pH, and glucose concentration on fermentation using *S. cerevisiae* DBTIOC S24; (A) at different temperatures (25 - 42°C) with initial glucose concentration 180 g L^{-1}, pH 5.5; (B) at different pH (3 - 7) with initial glucose concentration 180 g L^{-1}, temperature 30 °C and (C) at different initial glucose concentration (60 - 180 g L^{-1}) with pH 5.5, 30 °C. Glucose consumption (solid bar) and ethanol concentration (unfilled bar)

Fig. 3 Fermentation Profile of *S. cerevisiae* DBTIOC S24 at different temperature i.e. 30 °C (A-B), 37 °C (C-D) and 42 °C (E-F) using 180 g L^{-1} initial glucose concentration at pH 5.0±0.2 in absence (A, C and E) and presence (B, D and F) of inhibitors (acetic acid 2.5 g L^{-1}; formic acid 0.25 g L^{-1}; 5-HMF 0.75 g L^{-1} and furfural 0.6 g L^{-1}) DCW: Dry cell weight

Fig. 4 Ethanol fermentation profile of *S. cerevisiae* DBTIOC S24: (A) using rice enzymatic hydrolysate in SHF and (B) using fermentation media with glucose (80 g L^{-1}) as a control, at 30 °C (solid line) and 42 °C (dotted line). The hydrolysate was used without filtration, sterilization or detoxification

Fig. 5 Fermentation profile of *S. cerevisiae* DBTIOC S24 in SSF of dilute acid pretreated rice straw slurry at (A) 20 % and (B) 25 % solid loading. 20 FPU enzyme per g biomass was added in starting (BPS: before pre-saccharification i.e., 3 h)



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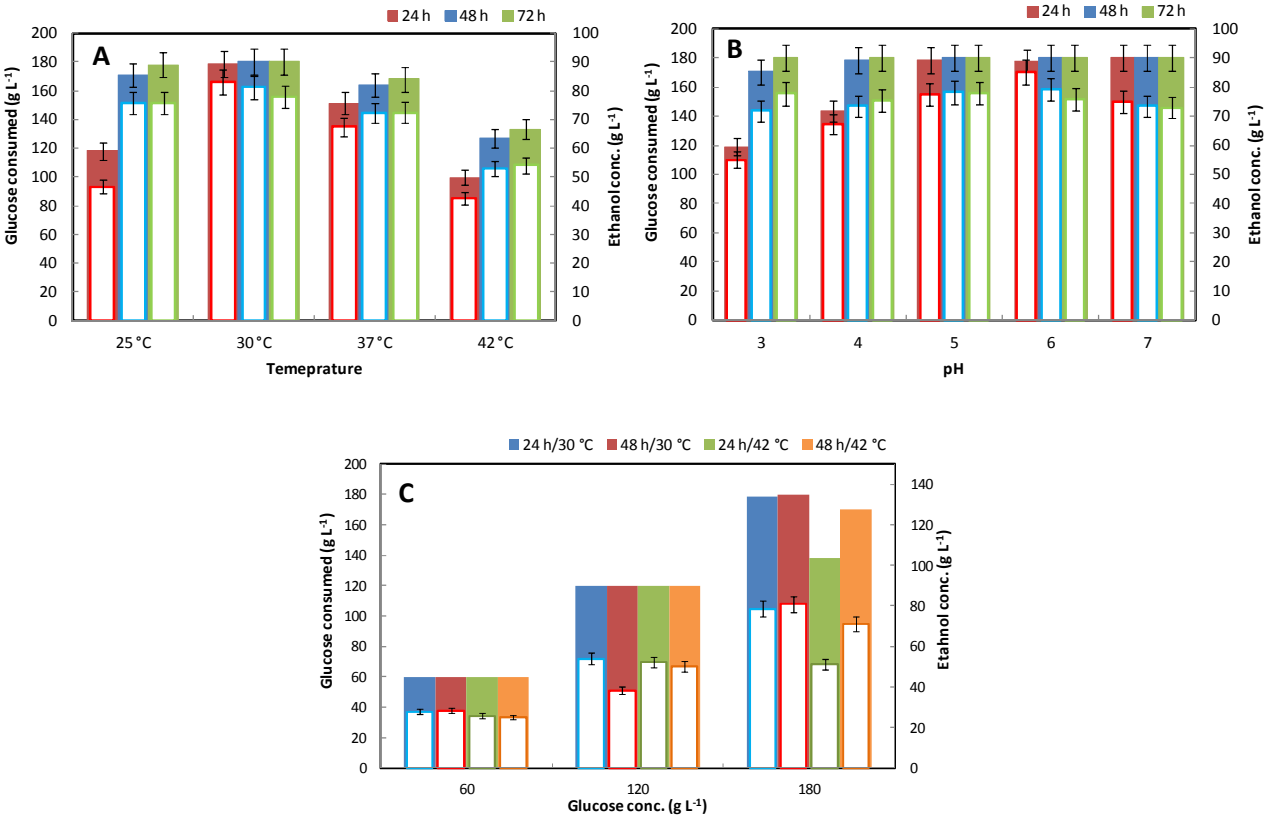

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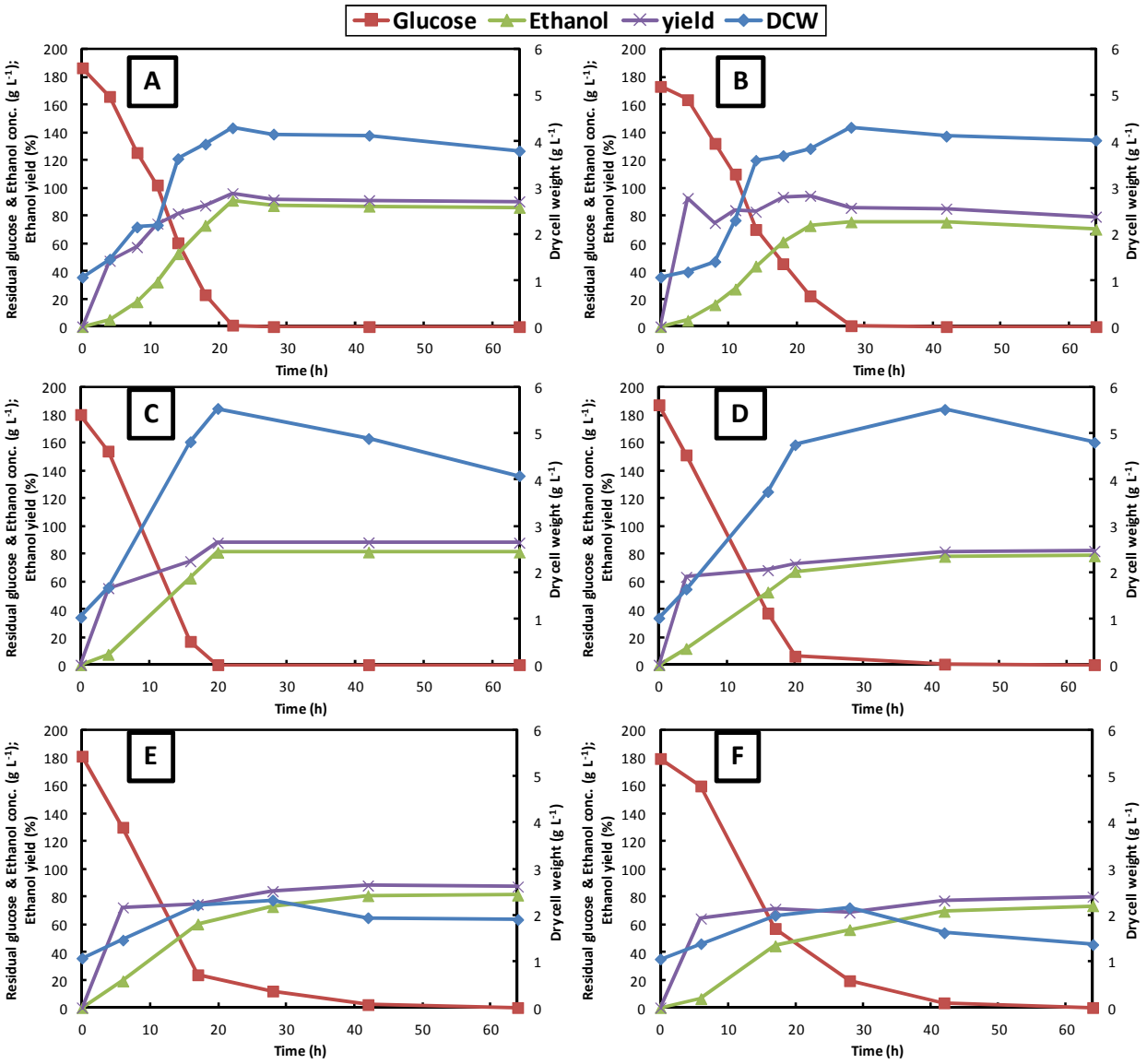

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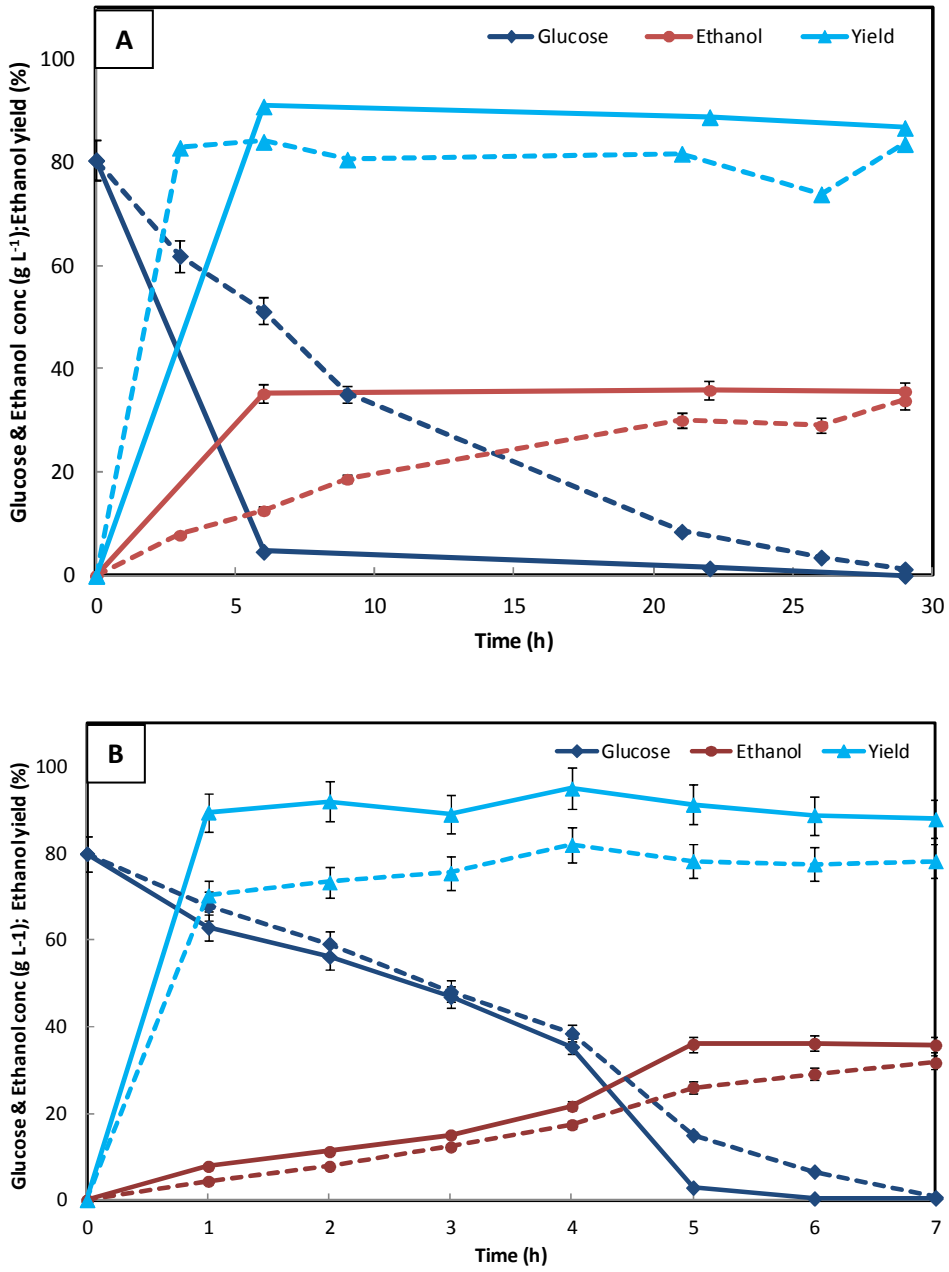

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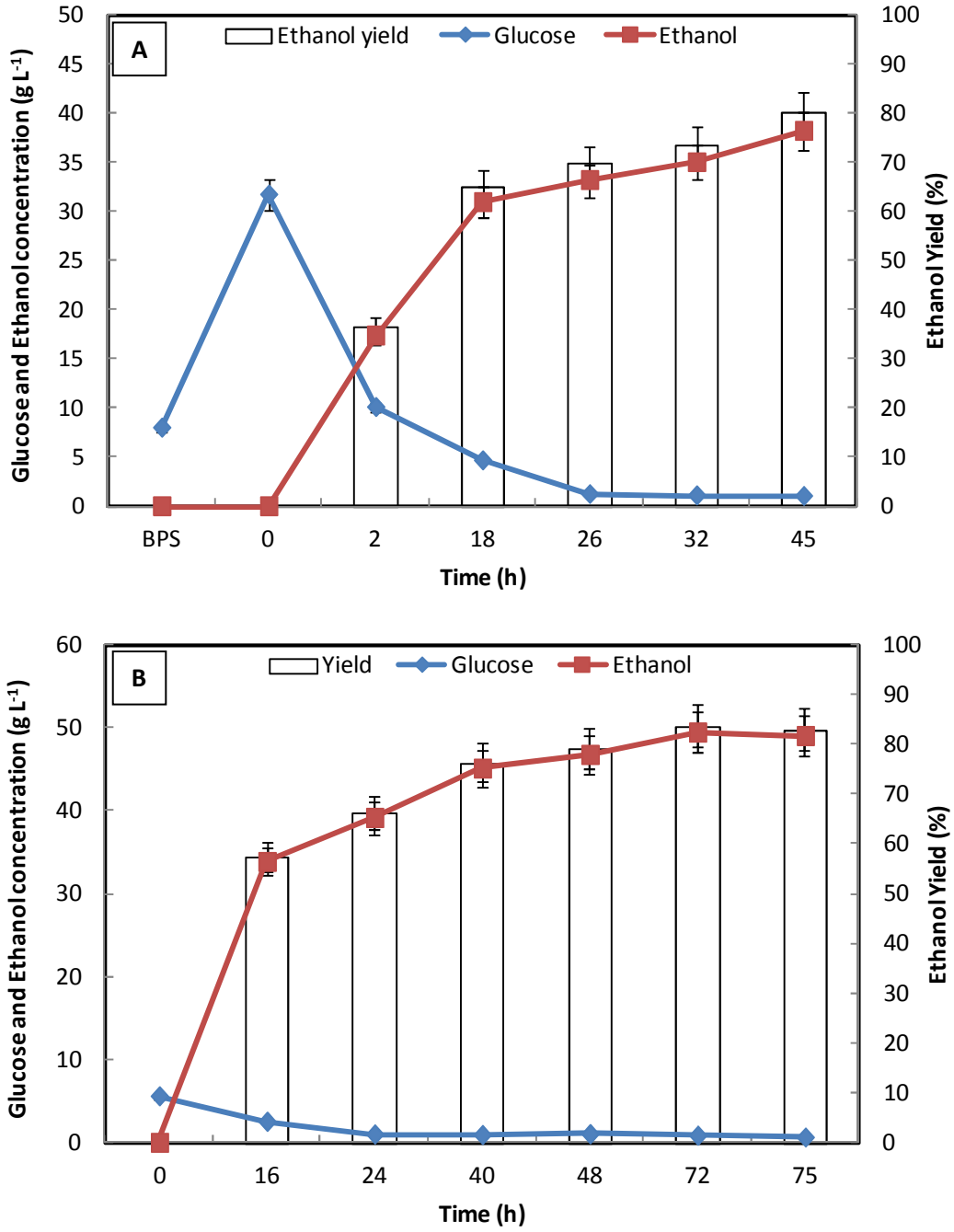

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