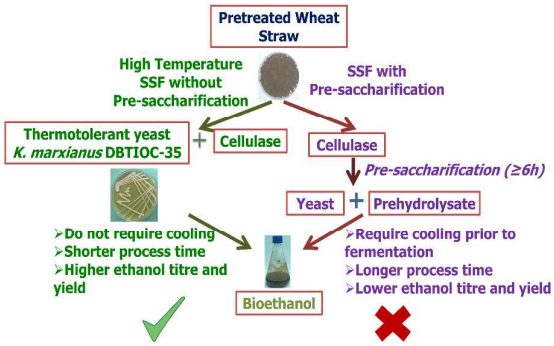
RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Application of thermotolerant yeast *Kluyveromyces marxianus* DBTIOC-35 in SSF decreases overall process time, and increases yield by allowing elimination of presaccharification step and use of high biomass concentration.



Second generation bioethanol production at high gravity of pilot-scale pretreated wheat straw employing newly isolated thermotolerant yeast *Kluyveromyces marxianus* DBTIOC-35

Jitendra Kumar Saini[1]*, Ruchi Agarwal[1], Alok Satlewal[1], Reetu Saini[1], Ravi Gupta[1], Anshu Mathur[1] and Deepak Tuli[1]*

[1]*DBT-IOC Centre for Advanced Bio-Energy Research, R & D Centre, Indian Oil Corporation Ltd., Sector-13, Faridabad-121007, Haryana, India*

Correspondence:

E-mail: tulidk@indianoil.in; Ph. +91 129 2285340; +91 129 2294273 (D. Tuli)

E-mail: me_jk@rediffmail.com; Ph. +91 129 2294345; (Mob) +91 99719 58118 (J.K. Saini)

Abstract

Second-generation bioethanol production was studied by a newly isolated thermotolerant yeast strain at 42 °C and above using pilot-scale dilute acid pretreated wheat straw (WS) as feedstock. This strain was identified as *Kluyveromyces marxianus* DBTIOC-35 by biochemical characterization as well as molecular phylogenetic analysis of ITS-5.8S rRNA gene and D1/D2 domain of the 26S rRNA gene after PCR amplification and sequencing. Simultaneous saccharification and fermentation (SSF) at 42 and 45°C using 10 % biomass loading resulted in ethanol titer of 29.0 and 16.1 g/L, respectively. At 42°C ethanol productivity was higher during SSF (0.92 g/L/h) than separate hydrolysis and fermentation (SHF) (0.49 g/L/h) at 20% biomass loading. Results indicated that at 20% biomass loading, SSF without pre-saccharification led to more ethanol production (66.2 g/L with 83.3% yield) at a faster rate than SSF with pre-saccharification (PSSF) which produced ethanol titer of 61.8 g/L, 77.7% yield and productivity of 0.86 g/L/h. Based on these findings, application of newly isolated yeast *K. marxianus* DBTIOC-35 in SSF of lignocellulosic biomass can eliminate pre-saccharification step which is a novel advantage of thermotolerant yeasts in terms of cutting down the overall biomass to bioethanol process time and enhancing bioethanol titer, yields and productivities.

Keywords: SSF; thermotolerant yeast; *Kluyveromyces*; bioethanol; wheat straw; dilute-acid pilot scale pretreatment

Introduction

Bioethanol production is considered a green technology for mitigating greenhouse gas emission and partial replacement of fossil transportation fuels (such as gasoline). [1, 2] Bioethanol production has increased enormously during the past decade. Bioethanol made from lignocellulosic biomass (such as corn stover, rice straw, sugarcane bagasse, wheat straw etc.) is termed second generation (2G) bioethanol in contrast to first-generation bioethanol that is derived from sugar and starch based materials (such as sugarcane and grains).[3] Feedstock for 2G bioethanol are abundant, renewable, cheaper and do not compete with food resources. Of the various agricultural lignocellulosic wastes wheat straw has the second largest availability of approximately 354.34 million tons with an estimated bioethanol production potential of at least 104 Giga litres.[4] Due to food versus fuel concerns developing nations like India cannot utilize food resources (such as grains or sugar) for biofuel production and must rely upon renewable lignocellulosic biomass.

2G bioethanol production from lignocellulosic feedstock involves pre-treatment, saccharification, fermentation and ethanol recovery. Pre-treatment helps in decreasing the crystallinity of cellulose, increasing biomass surface area, removing hemicellulose, and breaking lignin seal to make cellulose more accessible to cellulase enzymes. The biochemical route for bioethanol generation relies upon conversion of cellulose (and hemicelluloses) into fermentable sugars using (hemi-) cellulolytic enzymes followed by fermentation of sugars into ethanol via separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF).[5] Some bottlenecks to economically viable biochemical conversion processes include better pre-treatment process to increase enzyme accessibility, the cost and efficiency of cellulase enzymes, blending of cellulase enzymes to achieve better hydrolysis,[6] process and strain engineering to improve xylose and glucose co-fermentation[5] etc. In its original form, the batch SSF reactor initially

contains substrate, enzymes as well as yeast cells at the intended concentrations.[5] Major drawback of SSF process is mis-match of optimum temperatures of enzymes and most of fermenting microorganisms (e.g. the optimum temperatures are approximately 50 °C and 30-35 °C, respectively). Therefore, several modifications in SSF have been suggested, such as inclusion of a pre-saccharification step at optimum enzyme temperature to allow sugar hydrolysis before cooling down to yeast optimum fermentation temperature,[1,7] varying the temperature during SSF and use of fed-batch approach for biomass addition.[8-9] Though these alterations can enhance the ethanol titer, the overall fermentation time is also increased resulting in low productivities that are undesirable for commercially viable process. Thermotolerance of the fermenting microorganisms is vital aspect in this regard.[10] Thermotolerant yeasts strains that have been evaluated in SSF process include *Kluyveromyces marxianus*, *Fabospora fragilis, Saccharomyces uvarum, Candida brassicae, C. lusitaniae,* etc. Use of thermotolerant yeasts can allow fermentation at temperatures closer to the optimal range (around 50°C) of the enzymes. Therefore, high degree of hydrolysis and subsequent higher ethanol yields can be obtained within lower time making SSF process more efficient. Other benefits of bioethanol fermentations at high temperature (≥40°C) are faster fermentation rates, reduction in cooling costs and prevention of contamination.[11] Therefore, application of thermotolerant microorganisms that can ferment high ethanol concentration can be a major development in SSF process. Higher temperatures are reported to negatively affect yeast viability and growth[12] which makes the search for better thermotolerant microorganisms even more necessary.

The aim of the current study was to enhance bioethanol production using dilute acid pilot scale pretreated wheat straw. To achieve this, indigenous strain of thermotolerant yeast was evaluated for fermentation at ≥42°C under different process configurations. Though there are few reports on use of thermotolerant yeasts, to the best of our knowledge

no comparable literature has highlighted the importance of thermotolerant yeasts in decreasing the lignocellulose to ethanol bioconversion process time by complete elimination of presaccharification step and enhancement of productivities during SSF and only fewer studies have utilized pilot scale pretreated biomass in SSF for ethanol production.

Experimental

Materials

Peptone, yeast extract, D-glucose and all other medium components used in this study were procured from HiMedia, India. Antibiotics ampicillin and streptomycin were purchased from MP Biomedicals, USA and Sigma-Aldrich, USA, respectively. SacchariSeb C6, a commercial cellulase powder, was purchased from Advanced Enzymes Inc, India. Wheat straw was obtained from local farmers.

Fermenting microorganism and culture conditions

Whey samples collected in sterile bottles from local dairies at Noida, India were used for isolation of thermotolerant yeasts by enrichment culture technique using YPD medium (yeast extract - 1%, peptone - 2%, glucose - 5%, filter sterilized ampicillin and streptomycin - 50 mg/L each). After enrichment for 48 h, the developed yeast colonies were evaluated qualitatively and quantitatively for bioethanol production at ≥ 40°C. The most promising isolate DBT-IOC-35 was selected for this study on basis of maximum ethanol titer, yield and ethanol efficiency during fermentation on glucose at 42°C (not shown). This strain was maintained at 4°C on YPD agar slants and at -20°C as glycerol stock for short and long term storage, respectively. Yeast fermentation media (YFM) used for bioethanol production contained (%): KH_2PO_4, 0.1; NH_4Cl, 0.03; $MgSO_4.7H_2O$, 0.2

Yeast extract, 0.5 and Peptone, 0.5; pH 5.0. For inoculum development, yeast cells were grown at 42°C for 24 h in 150 mL YPD medium. Yeast cells were recovered after centrifugation at 5000 rpm for 20 min, washed twice in saline and used as inoculum (approximately 1 g/L, dry wt.) for bioethanol production.

Biochemical characterization and molecular phylogenetic analysis

The yeast cell morphology was examined by bright field microscopy (Nikon eclipse Ni, Tokyo, Japan). Biochemical profile of the strain was studied on basis of carbohydrate utilization characteristics using a HiCarbohydrate^{TM} Kit (HiMedia Lab. Pvt. Ltd, India) by following manufacturer's instructions. For molecular phylogenetic analysis, the genomic DNA was extracted and purified from overnight grown culture of strain DBT-IOC-35 using Rapid Yeast Genomic DNA Extraction Kit (Bio Basic Inc., Ontario, Canada). The internal transcribed spacer region (ITS) 1 - 5.8S rRNA gene – ITS 2 region and D1/D2 domain of the 26S rRNA genes were amplified by polymerase chain reaction (PCR) using the primer pairs ITS1, ITS4 and NL1, NL4 respectively. [13, 14] The PCR products were purified using HiPurA PCR Product Purification Kit (HiMedia, India) and then sequenced by Macrogen Inc. (Korea) using an automated Applied Biosystems® 3730/3730xl DNA analyzer. The phylogenetic analyses were performed using the program CLUSTALW. Phylogenetic tree were constructed using the neighbour-joining method with the program MEGA4 and boot-strap analysis based on 1000 replicates.[15]

Pilot scale dilute acid pretreatment and composition analysis of biomass

Pretreatment of WS was carried out in 250 kg biomass/day capacity continuous pilot-scale pretreatment reactor. This multipurpose pilot plant is capable of pre-treatment operation for multiple feed (wheat straw, rice straw, sugarcane bagasse, cotton stalk, corn stover, woody biomass, etc.) under a wide range of operating conditions and pre-treatment

chemicals (acid and alkali). Wheat straw was milled to 4-5 mm; air dried and then soaked in the acid solution (2.5%, w/w) for 30 min in a soaking chamber specially equipped with spray and circulation of acid solution. The wet biomass, after soaking was hung for 2 h and further pressed for 15 min at a pressure of up to 100 Bar in a hydraulic filter press to remove water. Then biomass was fed into the reactor at a rate of 10 kg/h and treated at 160°C and 6 bar with a residence time of 15 min. Residence time was controlled by the screw speed of the reactor. The pre-treated biomass slurry was collected in the slurry tank, cooled and then transferred through a peristaltic pump to a high speed centrifuge for separating solids (cellulose and lignin) and liquid (hemicelluloses).[16] Composition analysis of untreated (UWS) and pretreated wheat straw (PWS) was carried out by following the procedure of Sluiter *et al.*[17] After thorough washing and removal of extra water by pressing, moisture content of PWS was determined using MA150 electronic moisture analyzer (Sartorius weighing technology GmbH, Gottingen, Germany).

Enzymatic saccharification of biomass

Enzymatic saccharification of PWS was carried out in 500 mL capped flasks containing 200 mL reaction volume at 150 rpm. Influence of various parameters such as different biomass loadings (5 to 20%, wt.), temperature (30 to 50°C) and enzyme dosage (10 to 60 filter paper units (FPU)/g biomass) on enzyme hydrolysis was studied. The initial pH of hydrolysis slurry was maintained at 5.0 using 1 M citrate buffer.

Separate hydrolysis and fermentation (SHF)

For separate hydrolysis and fermentation studies, enzyme hydrolysis was carried out for 72 h at 50°C using different loadings of PWS (10, 15 and 20%, wt.) as described earlier. Sterilized YFM and yeast inoculum were added after enzyme hydrolysis and flasks

were incubated further at 42ºC for 72 h. Samples were withdrawn every 24 h for estimation of ethanol and sugars.

Simultaneous saccharification and fermentation (SSF)

SSF experiments were performed in 500 mL capped Erlenmeyer flasks and the process was optimized for most favourable conditions of temperature (42 and 45ºC), substrate concentration (10, 15, 20 and 25 % wt.) and enzyme dosage (10, 20, 30, 40, 50, 60 FPU/g biomass). After addition of YFM and yeast inoculum, flasks were incubated at 42ºC and 150 rpm for 72 h. Samples were withdrawn at regular intervals for estimation of bioethanol and sugars. SSF ethanol results were reported as percentage of theoretical yield, considering the availability of all potential glucose in biomass for fermentation and a theoretical yield of 0.51 g ethanol/g glucose.[16]

Pre-saccharification and simultaneous saccharification and fermentation (PSSF)

The PSSF experiments were carried out in 500 mL flasks and included 6 h enzymatic pre-saccharification step at 50ºC followed by SSF for 72 h. Prior to the start of the fermentation, temperature was lowered to 42ºC and nutrients and yeasts were added. Three different biomass loadings (10, 15 and 20%, wt) were investigated. Samples were withdrawn after 6 h pre-saccharification (0 h fermentation) and then every 24 h during SSF, and were analysed for ethanol and glucose content.

Analytical methods

Total reducing sugars during enzyme hydrolysis were estimated by DNS method[18] by reading absorbance at 540 nm using UV-VIS Spectrophotometer (UV 1800 Spectrophotometer, Shimadzu, Japan). Sugars (glucose, xylose, cellobiose, galactose, arabinose, and mannose), glycerol and inhibitors in pretreatment slurry were analyzed by

high performance liquid chromatography (HPLC, Waters, USA) equipped with a refractive index detector and Aminex HPX-87H column 300 mm×7.8 mm ID (Bio-Rad Labs, Hercules, CA) . For ethanol estimation Clarus-680 Gas Chromatograph (Perkin-Elmer, USA) fitted with a 30 m long Carbowax-PEG column (Perkin-Elmer, USA) having inner diameter of 0.32 mm was used. The conditions used for HPLC and GC analysis and cellulase enzyme assays were same as described earlier.[16] All samples were appropriately diluted and filtered through a 0.22 μm disc-filter prior to analysis. All experiments were conducted in triplicates and average values are shown.

Calculations

Hydrolysis yield (%) =

$$[\text{Total Sugars (g/L)} / (0.511 * f * \{\text{Biomass}\}_0 * 1.111)] * 100 \quad (1)$$

$$\text{Ethanol titer (g/L)} = EtOH_t - EtOH_0 \quad (2)$$

$$\text{Ethanol yield (\%)} = [\text{Ethanol titer} / (0.511 * f * \{\text{Biomass}\}_0 * 1.111)] * 100\% \quad (3)$$

$$\text{Ethanol productivity (g/L/h)} = \text{Ethanol titer} / t \quad (4)$$

Where, $(EtOH_t - EtOH_0)$ indicates total ethanol produced during fermentation (g/L) run obtained by calculating the difference between ethanol produced initially (0 h fermentation) and after time 't', $\{\text{Biomass}\}_0$ is the initial dry biomass concentration (g/L), f is cellulose fraction of dry biomass, 0.511 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. [16]

Results and Discussion

Identification and characterization of strain DBTIOC-35

One of the desired characteristics for development of an efficient bioethanol fermentation process is the application of yeast strains that can endure high stress of temperature or ethanol during fermentation process. Thus, isolation and characterization of thermotolerant and stress tolerant yeasts is critical in improving the bioethanol production processes via SSF.[10] For this study, thermotolerant yeasts were isolated and qualitatively screened for growth and fermentation at 42°C and 45°C. Strain DBT-IOC-35 exhibited best ethanol fermentation characteristics (0.49 g ethanol/g glucose) under shake flask conditions (data not shown). This isolate had typical yeast-like colonies on YPD agar plates and reproduced by budding. The colonies were observed as single, or in pair. It fermented dextrose, lactose, fructose, galactose, and raffinose efficiently whereas utilization of xylose, arabinose, mannose, glycerol, α-methyl-d-glucoside, xylitol showed variable pattern during carbohydrate utilization test. This strain could not utilize citrate and malonate as sole carbon sources, but hydrolysed esculin and metabolized ONPG, thus indicating β-galactosidase activity. Biochemical profile of the strain matched closely to the reference strain *K. marxianus* MTCC-4136. Molecular phylogenetic analysis of ITS-5.8S rRNA gene and D1/D2 domain of the 26S rRNA gene of DBTIOC-35 revealed maximum similarities to *K. marxianus* Kw1696 (HE650694) (Fig. S1) and *K. marxianus* DX3-3 (GU565206) (Fig. S2). Therefore, isolate DBTIOC-35 was finally identified as *K. marxianus.* ITS-5.8S and 26S rRNA gene sequences were submitted to NCBI GenBank under accession numbers KP192932 and KP192933, respectively. Many studies have reported the abilities of *K. marxianus* strains to grow and ferment rapidly at temperatures ≥ 40 °C[10] but the reported ethanol yields or concentrations are mostly low. Therefore, to explore full potential of this strain in bioethanol applications, it was evaluated in SHF and SSF.

Biomass pre-treatment and composition analysis

Composition analysis (cellulose, hemicelluloses and lignin) of extractive free UWS and PWS indicated substantial removal of hemicelluloses (from 26.3% in UWS to 3.8% in PWS) and relative increase of 3.9% in the lignin content. Cellulose content also increased from 51.2% to 69.8% after pre-treatment. These findings are in accordance with that of Chen *et al.*[19] The dilute sulfuric acid pretreatment at high temperature hydrolyzes hemicellulose into monomeric sugars (xylose, arabinose, galactose, glucose, and mannose) and oligomers. Small amount of lignin is also depolymerized during acid pretreatment and might re-condense to form an altered lignin polymer. Removal of hemicellulose increases surface area and pore volume of the substrate which enhances the yield and rate of enzymatic hydrolysis of the cellulose rich biomass.[20] After pretreatment, most of the soluble monomeric and/or oligomeric sugars and inhibitors like furfural, hydroxyl-methyl furfurals and acetic acid remained in the liquid supernatant obtained after centrifugation. In most of the studies on bioethanol production, dilute acid pretreatment of biomass is generally carried out at a lab scale (mostly autoclaving the biomass containing shake flasks or bottles) with lower volume of pretreated biomass. In this study, dilute acid based pretreatment was carried out in a continuous pilot scale pretreatment reactor and this pretreated wheat straw was further used for enzymatic saccharification and bioethanol production studies.

Effect of enzyme dosage, biomass loading and temperature on enzymatic hydrolysis of PWS

Fig. 1a and 1b show the effect of enzyme dosage on hydrolysis of PWS at 50 °C and 42 °C, respectively. Pretreated WS was made free of sugars and inhibitors (phenolics, acetic acid, furfural etc.) due to their known detrimental effects on cellulase enzyme.[21] Hydrolysis yield increased with increase in the enzyme dosage at both temperatures. The yields at 42°C were lower than that at 50°C. Hydrolysis yield of 85% was attained by

using enzyme dosage of 45 FPU/g at 42°C in comparison to 30 FPU/g at 50°C. Therefore, 45 FPU/g PWS was chosen for further investigation. Karthika *et al.*[22] previously reported an enzyme dose of 30 FPU/g to obtain 80% hydrolysis within 48 h. PWS hydrolysis at different biomass loadings at two different temperatures is depicted in Fig 1c and 1d. At both temperatures, little or no difference in hydrolysis yield was observed at 5 and 10% biomass loading (dry wt). But there was reduction in hydrolysis yield at higher solid loadings (15% to 20%) which was in accordance with Oloffsson *et al.*[5] This reduction in hydrolysis yield may be either due to increased viscosity resulting in mass transfer limitations or mixing difficulties or due to feedback inhibition on enzyme. However, use of high solids can significantly improve ethanol concentration in fermentation broth and is desirable for reducing ethanol recovery cost.[23] Influence of temperature on hydrolysis of PWS at 10% solid loading and enzyme dose of 45 FPU/g is shown in Fig. 2. As much as 83% and 90% hydrolysis yields were obtained at 24 h at 42°C and 45°C, respectively. These conditions were chosen for SSF experiments. The enzymatic hydrolysis at higher temperatures would potentially reduce the reaction time but cellulase system is rapidly inactivated above 45°C. Therefore, to increase saccharification and subsequent ethanol fermentation, SSF can be adopted since the sugars released by enzymatic hydrolysis are immediately consumed by the yeast and therefore feedback inhibition on enzyme is potentially avoided. These points were taken into consideration during SSF experiments in this study.

Bioethanol production employing thermotolerant yeast DBTIOC-35

Both SHF and SSF are established processes for ethanol production from lignocellulosic biomass. Major advantage of SHF is that both hydrolysis and fermentation steps can be run separately under their optimal conditions at 50°C and 30°C, respectively. SSF on the other hand is usually carried out at 35°C at pH 5. Based on previous reports on

SSF, biomass loading of 10% wt., yeast inoculums concentration of 1 g/L in SSF and YFM as fermentation medium were used during bioethanol fermentation in this study.[5, 24-27]

Separate hydrolysis and fermentation

For SHF experiments PWS was hydrolysed by the cellulase enzyme at three different loadings 10, 15, 20 % (wt.) for 72 h and the hydrolysate was subjected to fermentation after supplementation with YFM nutrients and yeast cells. Initial sugar concentrations, cellulose to glucose conversion after hydrolysis, ethanol yield (g ethanol/g glucose) and % yield (of theoretical maximum) obtained during SHF are presented in Table 1. Glucose in the SHF flasks (taking into account the dilution due to addition of medium and inoculum) ranged from 65.5 to 99.9 g/L, which corresponded to respective biomass concentrations. Increase in substrate concentration resulted in decreased sugar conversion and glucose yield. The ethanol yields (g/g) obtained during SHF were close to those of control fermentation experiments (0.49 g ethanol/g glucose) using synthetic media (data not shown), which indicated overall good fermentation performance of strain DBTIOC-35. In SHF10 flasks almost complete utilization of glucose along with ethanol concentration of 26.8 g/L was observed after 24 h. Residual glucose in SHF15 and SHF20 flasks indicated incomplete sugar utilization and decreased the ethanol yield. Ethanol productivity (initial 24 h) in all flasks was proportional to biomass concentration. In SHF20 flasks ethanol concentration increased, however, some of the glucose released during saccharification remained unutilized even up to 72 h indicating the decrease in cell viability. During SHF of lignocellulosic biomass, amount of ethanol produced in fermentation step mainly depends upon sugar concentration obtained after saccharification step. Therefore, to achieve higher ethanol concentrations, and reduce fermentation vessel

size and wastewater streams higher initial substrate concentrations are preferred during SHF.[7]

Simultaneous saccharification and fermentation (SSF)

SSF experiments were aimed at increasing the final ethanol concentration. The frequently reported main advantages associated with this process are prevention of end-product inhibition during enzymatic hydrolysis resulting in comparatively higher saccharification and ethanol production, use of a single vessel for saccharification and fermentation step, elimination of separation of residual biomass prior to fermentation.[26]

Effect of temperature on SSF

Fig. 3a and 3b depict ethanol concentrations and yields (% of theoretical ethanol) estimated at 16, 24, 48, 72, 96 and 120 h during SSF at 42ºC and 45ºC, respectively. Tolerance to high temperatures besides high ethanol and sugar tolerance is an important aspect for selecting robust bioethanol producing microorganisms. Therefore, bioethanol production via SSF was studied at higher temperatures (> 40°C) at 10% initial biomass loading. Maximum ethanol concentration of 29.0 and 16.1 g/L were achieved, corresponding to ethanol yields (percentage of theoretical) of 73% and 40.5% at 42 and 45ºC, respectively. SSF at 45ºC resulted in lower ethanol production than at 42ºC. However, the observed ethanol concentration was much higher than 6.18 g/L from rice cellulignin[11] and 10 g/L from wheat straw. [25] At this temperature, accumulation of sugars (glucose as well as some xylose) was noticeable and was mainly due to inhibitory or lethal effect of this temperature on yeast growth and metabolism. Lin *et al.,* [28] have reported 80% decrease in viability of *K. marxianus* as a reason for decreased ethanol fermentation at 45ºC. At both the temperatures used in this study, maximum ethanol production was observed between 72-96 h, 42ºC being the more favourable temperature in terms of

bioethanol titer, yield as well as productivity and was used further. Though there are a few reports on bioethanol fermentation at temperatures as high as 45ºC, but the obtained ethanol yields are inferior to that obtained at lower temperatures (42ºC or 37°C). Due to decrease in cell viability, ethanol production decreases considerably at higher temperatures. [10]

Effect of biomass loading on SSF

To further increase ethanol concentration in the fermentation broth, SSF process was carried out at high substrate loading. Fig. 3c, 3d and 3e show ethanol production during SSF at 42°C using different biomass loadings. After 72 h, highest ethanol yields at 20 and 25% biomass loadings were 83.3% and 67.9%, respectively. Substrate concentration of 25% hampered the ethanol yield. With increase in solid content, increased viscosity creates difficulty in stirring and limits the enzymatic hydrolysis of biomass possibly due to inhibition of binding of cellulase to substrate under such conditions.[7, 23] However, in present study, ethanol yield increased with increase in biomass loading from 10 to 20%, wt. in contrast to reports indicating decrease in ethanol yield above 10-15%.[5] One probable reason might be release of sugars in controlled manner (as no residual glucose was observed in the SSF medium) and immediate utilization and fermentation to ethanol by yeast. Detailed studies are needed to determine exact reasons for improvements in ethanol yields with increasing biomass loading up to 20%. Ethanol concentrations of 66.2 and 67.4 g/L were the maximum observed at 20 and 25% biomass loadings, respectively. Higher ethanol concentration obtained during SSF experiments might have been lethal under the process conditions (e.g. higher temperature) and possibly inhibited further ethanol production. This was supported by the accumulation of residual glucose when biomass loading of 25% was used. Moreover, very high dry matter content in the SSF process increased the viscosity, at least initially, and might inhibited even mixing of

slurry. Though fed-batch as well as pre-saccharification strategies can alleviate some of the problems associated with high DM concentrations, use of good bioethanol fermenting thermotolerant yeast strains such as *K. marxianus* DBTIOC-35 could provide a better alternate solution.

Effect of Pre-saccharification and simultaneous saccharification and fermentation (PSSF)

Ethanol production rate in SSF is governed more by cellulose hydrolysis rather than glucose fermentation.[23] But when the biomass concentration is increased in SSF, mixing of the solids is decreased. Therefore, one approach repeatedly employed is to carry out prehydrolysis or pre-saccharification of the biomass at optimum temperature of enzyme for some time prior to yeast inoculation. This makes the SSF slurry more fluid and easy to handle. Time course of ethanol production during PSSF at 42°C with different biomass loading has been shown in Fig. 4a-4c. Pre-saccharification step of 6 h at 50°C at different WS concentrations was evaluated during this study. The conditions for PSSF were chosen based on the obtained hydrolysis efficiency of more than 54% during enzymatic hydrolysis experiments (Fig. 1). The main aim of PSSF was to partially hydrolyse the cellulose to sugars prior to yeast addition so that ethanol production during initial phase of PSSF could be increased. The positive effect of PSSF on ethanol production during initial 24 h was evident in experiments at 15 and 20 % biomass loading. However, after 48 h both SSF and PSSH resulted in almost similar ethanol concentration and yields. After 72 h ethanol production was lower than SSF performed without any pre-saccharification step. Thus, the pre-saccharification step in comparison to SSF did not result in enhanced ethanol production by *K. marxianus* DBTIOC-35 in this study. Similar findings have been reported earlier for olive pruning biomass, corn stover and barley straw due to more enzyme deactivation at high temperature pre-saccharification step.[1, 23] This

might have decreased the ethanol yield by lowering the enzyme hydrolysis in the later stages of PSSF. Lower ethanol yields can also be a consequence of decreased water activity in the fermentation broth, especially at higher biomass loading or due to sudden osmotic changes in extracellular environment of the yeast which leads to osmostress response as a defense mechanism by making intracellular physiological changes such as glycerol formation.[29] Glycerol synthesis is an alternate means for NAD^+ replenishment during SSF process. Glycerol concentration in SSF broths were in the range of 2.1 to 3.56 g/L (not shown) and are comparable to 2.62 g/L reported previously during SSF of sunflower meal.[30] However, low extracellular glycerol may not rule out the formation of intracellular glycerol.

Effect of enzyme dosage on SSF

Total rate of ethanol production during SSF process is largely controlled by the enzymatic hydrolysis of the solid biomass. Previous studies on SSF at different enzyme loadings have exhibited strong positive correlation between enzyme loading and the overall ethanol yield.[5] However, SSF process can be made more cost-effective by decreasing the amount of cellulase needed to a level that doesn't compromise the ethanol production significantly. Therefore, SSF experiments were performed using different cellulase dosages at 20% biomass concentration (Fig. 5). At low enzyme loadings of 20 FPU/g, the overall ethanol productivity was not much affected and only a slight decrease from 0.87 g/L/h (at 40 FPU/g) to 0.82 g/L/h was observed. Similar previous findings have been attributed to better conversion of cellulose to glucose at higher cellulase loadings.[5] Application of higher enzyme dosages may seem uneconomical as such, but recycling of cellulase enzymes can be a potential strategy that can cut down the cost of enzymes used for biomass bioconversion. This will require more efforts in effective separation of enzymes dissolved in enzymatic hydrolysate and/or bound to left over solid residuals

mainly lignin, especially when using acid pretreated biomass.[31] Further decrease in enzyme loading to 10 FPU/g decreased ethanol productivity to 0.67 g/L/h which was much lower than at higher enzyme dosages. From process economy point of view, application of lower enzyme dose will definitely be more important in future in terms of cost savings and can make up the loss due to decreased productivity to some extent. However, choice of optimal enzyme dose will depend upon balanced trade-off between both the process economy and productivity.

Comparison of SHF, SSF and PSSF

Table 2 compares the ethanol production by thermotolerant yeast *K. marxianus* DBTIOC-35 using different process configurations SHF, SSF and PSSF at different loadings of PWS. In SHF with 10% biomass loading, faster initial (24 h) rate of ethanol production was observed than SSF or PSSF due to more availability of glucose initially. At solids loading above 10%, glucose availability increased in SSF but enzyme inhibition possibly became more dominant in SHF due to increased sugar release. As a result, initial rates of ethanol production for SHF and SSF differed at 20% biomass loading. The removal of sugar inhibition on enzyme in SSF enabled more sugar to be continuously released which in turn continuously increased ethanol production and resulted in higher ethanol titers. These findings are very well supported by the results of previous study.[32] Highest productivity were obtained during PSSF of PWS at biomass loadings ≥15% (wt.), however, these were only marginally better than that obtained during SSF without 6 h long pre-saccharification step. The overall productivities, as well as titer and yields during SSF process at different biomass loadings were better than other process configurations. During SSF and PSSF cellobiose and xylose concentration never exceeded 0.4 and 4.0 g/L, respectively and galactose, mannose and arabinose were not detectable.

Ethanol production by *K. marxianus* DBTIOC-35 during SSF has been compared with previous studies in Table 3. As can be seen, maximum overall ethanol productivity of 0.92 g/L/h obtained in this study at 20 % biomass loading was better than 0.39 g/L/h obtained by both Mohagheghi *et al.* [21] and Bollók *et al.,* [33] and 0.885 g/L/h reported by Jain *et al.*[34] Though there are some previous reports on SSF without pre-saccharification step, simple SSF process i.e. without any presaccharification or any other modifications (such as delayed temperature SSF or non-isothermal SSF) have not resulted in such high ethanol titres of 66.2 g/L (ethanol yield of 83.3%) as have been obtained in this study under high gravity SSF of pilot scale dilute acid pretreated wheat straw. Zhang *et al.,*[8] reported higher ethanol titer (84.7 g/L) than this study; however the process took longer (96 h) with comparatively lower ethanol yields and the biomass was added in fed batch process to increase biomass loading. Moreover, their process involved serial acid and alkali pretreatment which removes both hemicellulose and lignin but at the same time increases cost and environmental concerns.

Success of any SSF process depends upon balanced trade-off between optimal temperatures of enzymes and yeast which can be achieved by carrying out SSF process at temperatures above 40°C. Besides high fermentation rates, high temperature SSF may reduce the possible contamination by mesophilic microorganisms and enhance the hydrolytic enzyme activities. [35] Therefore, thermotolerant yeasts are highly desirable for cost-effective commercial production of second generation bioethanol as these can withstand higher temperatures encountered due to heat generation during large-scale industrial fermentations. The SSF process without pre-saccharification (as used in this study) has certain advantages such as, saving at least 4 h to 24 h of the overall process time, the energy used in heating and cooling during and after pre-saccharification, respectively, increased stability of the used enzyme during the process (in comparison to when higher temperature is used) etc. Ethanol concentrations obtained in the present study can be improved further by employing fed-batch SSF or temperature shift SSF process.

Conclusions

Thermotolerant yeast *K. marxianus* DBTIOC-35 capable of producing high titer ethanol above 40ºC with a maximum fermentation temperature of 45ºC has been successfully isolated. SSF of dilute acid pretreated WS at higher solids loadings at 42ºC using an efficient thermotolerant yeast can improve bioethanol titer and yield even without the pre-saccharification step and the overall biomass to ethanol conversion process time can be reduced. As far as our knowledge is concerned, ethanol titers and yields reported in this study are the highest when using dilute acid pretreated biomass in unmodified SSF process. Further studies on scale-up are underway and will certainly prove fruitful for development of a more sustainable and greener process for biomass to ethanol bioconversion.

Acknowledgement

All authors acknowledge Indian Oil Corporation R & D Centre and Department of Biotechnology, Govt. of India for providing financial support.

Bibliographic references and notes

1. K. Ohgren, J. Vehmaanperä, M. Siika-Aho, M. Galbe, L.Viikari and G. Zacchi, *Enzyme Microbial Technol.,* 2007, **40**, 607–613.
2. C.W. Murphy and A. Kendall, *GCB Bioenergy,* 2014, doi: 10.1111/gcbb.12204
3. J.K. Saini, R.K. Anurag, A. Arya, B.K. Kumbhar and L. Tewari, *Ind. Crops Prod.*, 2013, **44**, 211–219.
4. J.K. Saini, R. Saini and L. Tewari, *3 Biotech.*, 2014, doi: 10.1007/s13205-014-0246-5.
5. K. Olofsson, M. Bertilsson and G. Lidén, *Biotechnol. Biofuels*, 2008, doi: 10.1186/1754-6834-1-7.

6. M. Adsul, B. Sharma, R.R. Singhania, J.K. Saini, A. Sharma, A. Mathur, R. Gupta and D.K. Tuli, *RSC Advances*, 2014, **4**, 44726-44732.
7. L. Paulová, P. Patáková, M. Rychtera and K. Melzoch, *Fuel,* 2014, **122**, 294-300.
8. M. Zhang, F. Wana, R. Su, W. Qi and Z. He, *Bioresour. Technol.* 2010, **101**, 4959–4964.
9. D. Chu, J. Zhang and J. Bao, *Bioenerg. Res.,* 2012, **5**, 1020–1026.
10. D.A. Costa, C.J. de Souza, P.S. Costa, M.Q. Rodrigues, A.F. dos Santos, M.R. Lopes, H.L. Genier, W.B. Silveira and L.G. Fietto, *Appl. Microbiol. Biotechnol.,* 2014, **98**, 3829-3840.
11. R.C.A. Castro and I.C. Roberto, *Appl. Biochem. Biotechnol.,* 2014, **172**, 1553-1564.
12. L. Caspeta, Y. Chen, P. Ghiaci, A. Feizi, S. Buskov, B.M. Hallström, D. Petranovic, and J. Nielsen, *Science,* 2014, **346**, 75-78.
13. T.J. White, T. Bruns, S. Lee, and J. Taylor, in PCR Protocols, ed. M.A. Innins, D.H. Gelfand, J.J. Sninsky and T.J. White, Academic Press, San Diego, CA, pp. 315–322.
14. A.E.L. Hesham, V. Wambui, J.O.H. Ogola and J.M. Maina, *J. Genetic Eng. Biotechnol.,* 2014, **12**, 37–43.
15. K. Tamura, J. Dudley, M. Nei and S. Kumar, *Mol. Biol. Evol.,* 2007, **24**, 1596-1599.
16. R.R. Singhania, J.K. Saini, R. Saini, M. Adsul, A. Mathur, R. Gupta and D.K. Tuli, *Bioresour. Technol.,* 2014, **169**, 490-495.
17. J.B. Sluiter, R.O. Ruiz, C.J. Scarlata, A.D. Sluiter and D.W. Templeton, *J. Agric. Food Chem.,* 2010, **58**, 9043-9053.
18. G.L. Miller, *Anal. Chem.,* 1959, **31**, 426-428.
19. H. Chen, Y. Han and J. Xu, *Process Biochem.,* 2008, **43**, 1462–1466.
20. R. Kumar, A. Satlewal, S. Sharma, V. Kagdiyal, R.P. Gupta, D.K. Tuli and R.K. Malhotra, *J. Renewable Sustainable Energ.,* 2014, doi: 10.1063/1.4880215.

21. Mohagheghi, M. Tucker, K. Grohmann and C. Wyman, *Appl. Biochem. Biotechnol.*, 1992, **33**, 67–81.

22. K. Karthika, A.B. Arun and P.D. Rekha, *Carbohydr. Polym.,* 2012, **90**, 1038-1045.

23. P. Manzanares, M.J Negro1, J.M. Oliva, F. Saéz, I. Ballesteros, M. Ballesteros, C. Cara, E. Castro and E. Ruiz, *J. Chem. Technol. Biotechnol*., 2011, **86**, 881–887.

24. I.M. Banat, P. Nigam and R. Marchant, *World J. Microbiol. Biotechnol.*, 1992, **8**, 259–263.

25. Ballesteros, M.J. Negro, J.M. Oliva, A. Cabañas, P. Manzanares and A.M. Ballesteros, *Biochem. Biotechnol.,* 2006, **496**, 129–132.

26. E. Tomás-Pejó, J.M. Oliva, M. Ballesteros and L. Olsson, *Biotechnol. Bioeng.,* 2008, **100**, 1122–1131.

27. Edgardo, P. Carolina, R. Manuel, F. Juanita and J. Baeza, *Enzyme Microbial Technol.*, 2008, **43**, 120-123.

28. Y.S. Lin, W.C. Lee, K.J. Duan and Y.H. Lin, *Appl. Energ.,* 2013, **105**, 389-394.

29. J. Albertyn, S. Hohmann, J.M. Thevelein and B.A. Prior, *Mol. Cell. Biol.,* 1994, **14**, 4135–4144.

30. D. Camargo, S.D. Gomes and L. Sene, 2014. *Bioprocess Biosyst. Eng.* **37**, 2235-2242.

31. M.J. Taherzadeh and K. Karimi, *BioResources,* 2007, **2**, 707–78.

32. J.Y. Zhu, R. Gleisner, C.T. Scott, X.L. Luo and S. Tian, 2011. *Bioresour. Technol.* **102**, 8921–8929.

33. M. Bollók, K. Réczey and G. Zacchi, *Appl. Biochem. Biotechnol.,* 2000, **84-86**, 69–80.

34. R.K. Jain, D. Ghosh, D. Agrawal, S.K. Suman, D. Pandey, V.T. Vadde, A.K. Dixit, D.K. Adhikari and D. Dasgupta, *Biomass Conv. Bioref.,* 2013, doi: 10.1007/s13399-014-0143-5.

519 35. N.K. Pessani, H.K. Atiyeh, R. Wilkins, D.D. Bellmer and I.M. Banat, *Bioresour.*
520 *Technol.*, 2011, **102**, 10618–10624.
521 36. M. Boyle, N. Barron and A.P. McHale, *Biotechnol. Lett.,* 1997, **19**, 49–51.
522

Legends to Figures

Fig. 1. Enzymatic hydrolysis of PWS at different enzyme dose and 10% biomass loading at (a) 50˚C and (b) 42°C; and at different biomass loadings and 45 FPU/g at (c) 50˚C and (d) 42°C.

Fig. 2. Influence of temperature on biomass hydrolysis at 10% solid loading and enzyme dosage of 45 FPU/g.

Fig. 3. Ethanol production during SSF of WS at different temperatures and biomass loadings. SSF at (a) 42°C using 10% biomass loading; (b) SSF at 45°C using 10% biomass loading; SSF at 42°C using (c) 15% biomass loading; (d) 20% biomass loading; and (e) 25% biomass loading.

Fig. 4. Time course of ethanol production during PSSF at 42°C using (a) 10% biomass loading, (b) 15% biomass loading, and (c) 20% biomass loading.

Fig. 5. Ethanol production during SSF at different enzyme dosages. SSF was carried out at 42°C for 72 h using 20% biomass loading.

539 **Table 1. Initial glucose concentration, glucose conversion and ethanol yield during**
540 **SHF at different loadings of PWS*.**

Experiment ID	Substrate Concentration (wt.)	Initial glucose, g/L	Glucose Conversion	Time	Yield, g/g	Yield, % of theoretical (On basis of sugar utilized)
SHF10	10%	62.5	96.9%	24 h	0.47	91.5
				48 h	0.43	84.8
				72 h	0.43	83.5
SHF15	15%	87.4	90.0%	24 h	0.48	94.2
				48 h	0.43	84.3
				72 h	0.41	80.7
SHF20	20%	99.9	82.2%	24 h	0.49	95.4
				48 h	0.42	79.2
				72 h	0.40	78.0

541

542 *Enzymatic hydrolysis was done using enzyme dose of 45 FPU/g at 50°C and different
543 biomass loadings for 72 h and glucose conversion (after HPLC analysis) was calculated
544 from cellulose content of PWS on dry matter basis
545

546 **Table 2. Comparison of ethanol concentrations and yields (% of theoretical) obtained**
547 **during SHF, SSF and PSSF at different loadings of PWS.**

Biomass Loadings (%, wt.)	Final Ethanol concentration (g/L)			Yield (%)			Productivity (g/L/h)		
	SHF	SSF	PSSF	SHF	SSF	PSSF	SHF	SSF	PSSF
10%	26.3	29.0	27.6	67.8	73.0	66.5	1.11 (0.36)	1.06 (0.4)	1.06 (0.38)
15%	32.3	45.9	44.2	51.6	77.0	72.5	1.28 (0.45)	1.61 (0.64)	1.69 (0.61)
20%	35.2	66.2	61.8	39.8	83.3	77.7	1.31 (0.49)	2.17 (0.92)	2.27 (0.86)

548 Initial and overall ethanol productivities were calculated respectively, for 24 h and 72 h
549 (Values in parenthesis) fermentation period i.e after addition of yeast cells and does not
550 take into account the hydrolysis time (as in SHF or PSSF). SHF (72 h hydrolysis and 72 h
551 fermentation), SSF (72 h) and PSSF (6 h hydrolysis and 72 h fermentation) were carried
552 out at 42°C.

553 **Table 3. Comparison of bioethanol production via SSF process by *K. marxianus* DBTIOC-35 with previous studies.**

Microorganism	Substrate, Pre-treatment and solids loading (%, wt.)*	Enzyme Dosage	Fermentation Temperature and Time	Ethanol titer, g/L; yield, % (or g/100g); productivity (g/L/h)	Ref.
S. cerevisiae DQ1	A, CS, 30%	10-15 FPU/g DM Commercial cellulase	40°C	48 g/L; 65.6 %	**Chu *et al.*,**[9]
S. cerevisiae	A, WS, 20%	0.24 g Cellulase 150L/g cellulose	37 °C, 144 h	57 g/L; 80%; 0.39 g/L/h	**Mohagheghi *et al.*,**[21]
K. marxianus Y.00243	SE spruce, 5%	37 FPU/g+38 BGLU/g of commercial enzymes	42°C, 23 h	9.1 g/L; 14 g/100 g DM; 0.39g/L/h	**Bollók *et al.*,**[33]
K. marxianus CECT *10875*	SE WS, 10% WIS	15 FPU/g Celluclast 1.5 L + 12.6 IU/g Novozyme 188	42°C, 72 h	70%; 12 g ethanol/100 DM	**Ballesteros *et al.*,**[25]
Dry yeast (*S. cerevisiae*)	A+B, CC, 19%	30 FPU/g glucan Commercial cellulase	37°C, 96 h	62.7 g/L; 81.2; 0.65 g/L/h	**Zhang *et al.*,**[8]
Dry yeast (*S. cerevisiae*)	A+B treated CC, (19% initial + 6% after 4 h	30 FPU/g glucan Commercial cellulase	37°C, 96 h	84.7 g/L; 79.6%; 0.88 g/L/h	**Zhang *et al.*,**[8]
K. marxianus DBTIOC-35	A, WS, 20%	SaccariSeb C6 45 FPU/g	42°C, 72 h	66.2 g/L; 83.3%; (33.1g/100 g DM); 0.92 g/L/h	**This study**
K. marxianus DBTIOC-35	A, WS, 25%	SaccariSeb C6 45 FPU/g	42°C, 72 h	67.4 g/L; 67.9% (33.7g/100 DM); 0.94 g/L/h	**This study**
K. marxianus NRRL Y-6860	A, RS cellulignin	25 FPU/g Cellubrix and 25 IU Novozyme 188	45°C, 12 h	6.18 g/L; 47%; 24 g/100g	**Castro & Roberto,**[11]
Kluyveromyces sp. IIPE453	A, RS , 10%	1% v/w SacchariSeb C6	45°C,	17.7 g/L; 0.885 g/L/h	**Jain *et al.*,**[35]
K. marxianus IMB3	B, WS, 6%	2% (v/v) commercial cellulase	45°C, 60 h	3.6 g/L; 48%; 20g/100g WS	**Boyle *et al.*,**[36]
K. marxianus DBTIOC-35	A, WS, 10%	SaccharSeb C6 45 FPU/g	45°C, 72 h	16.1 g/L; 31.5%; 0.52g/L/h	**This study**

554

555 *CS = corn stover; WS= wheat straw; RS= Rice straw; WIS= water insoluble solids; A= acid pre-treatment; B= Alkali pre-treatment; A+B=Acid and alkali

556 combined pre-treatment; SE=steam explosion preachment

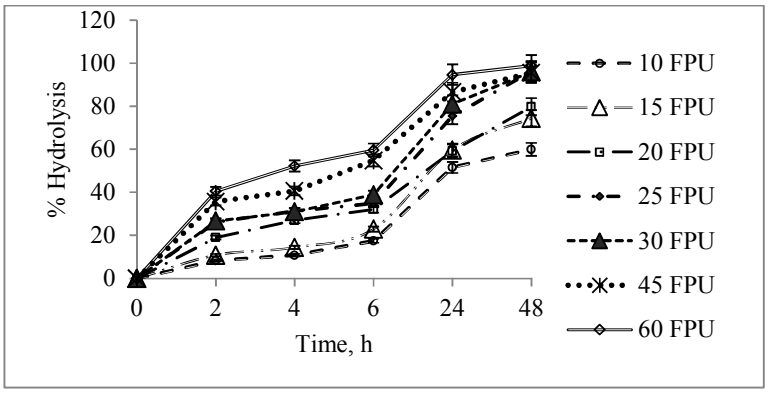


Fig. 1a.

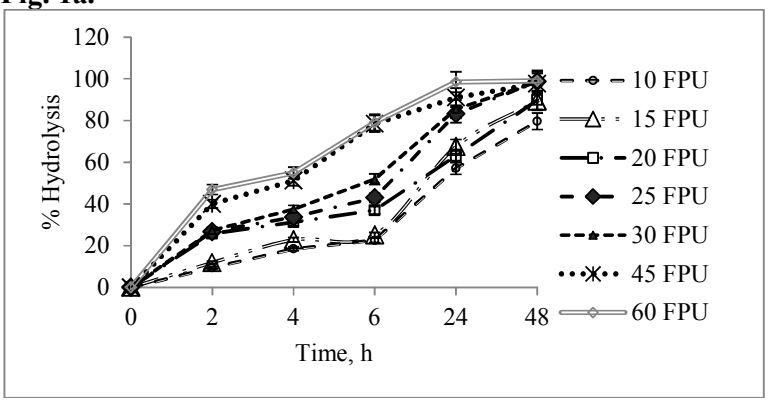


Fig. 1b.

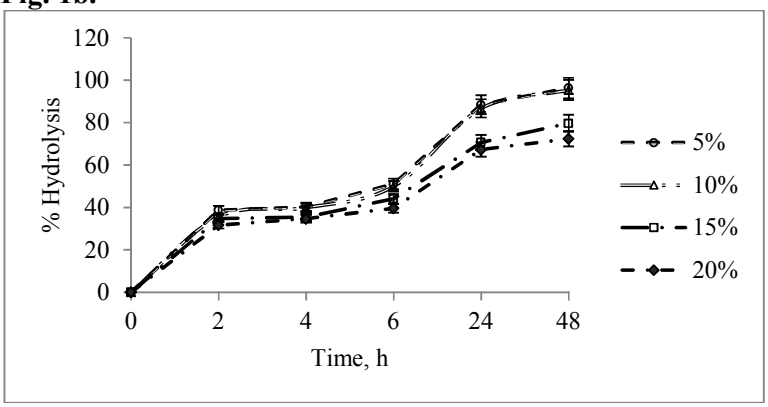


Fig. 1c.

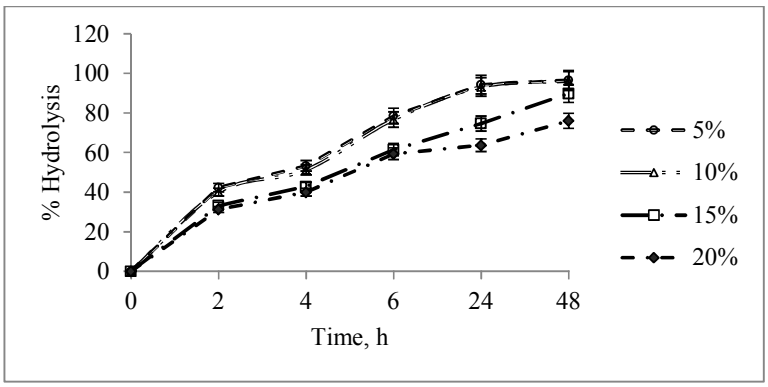


Fig. 1d.

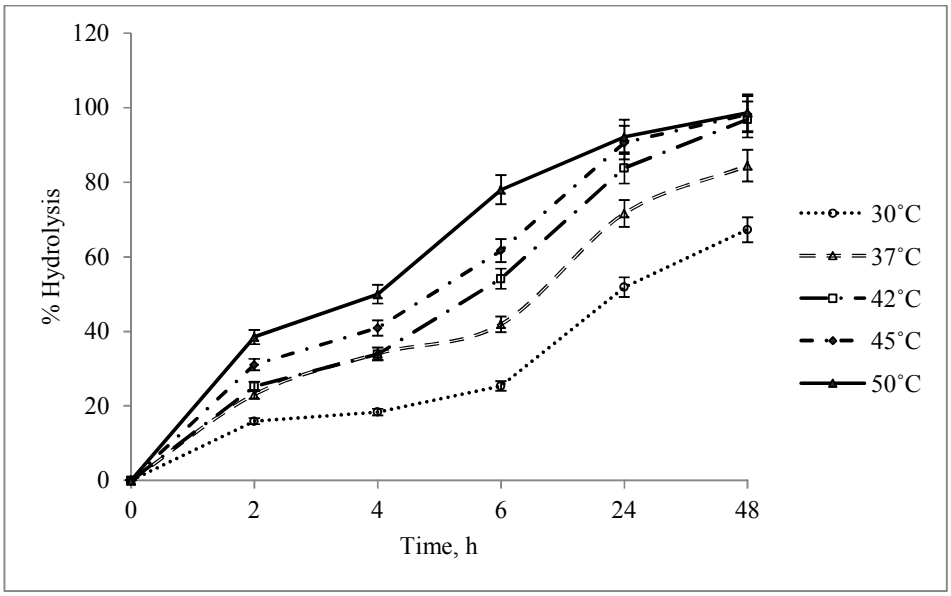


Fig. 2.

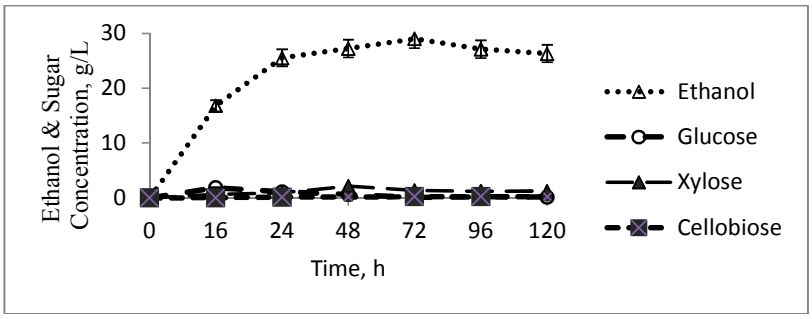


Fig. 3a.

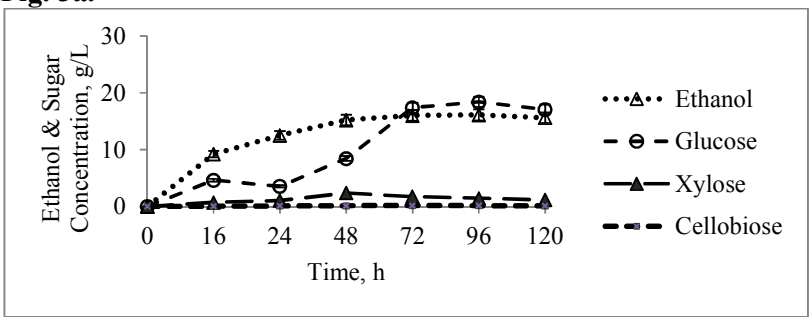


Fig. 3b.

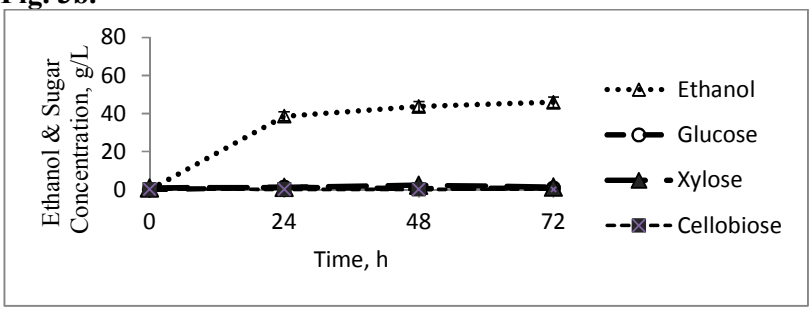


Fig. 3c.

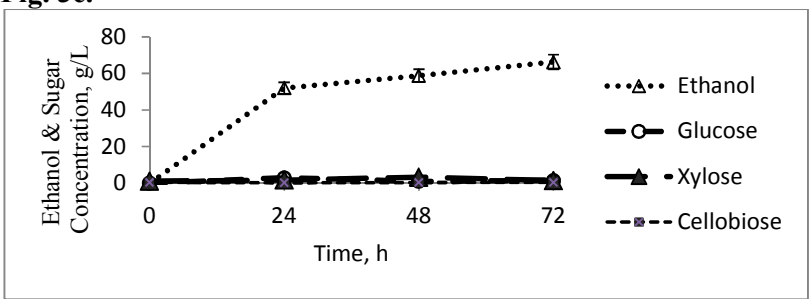


Fig. 3d.

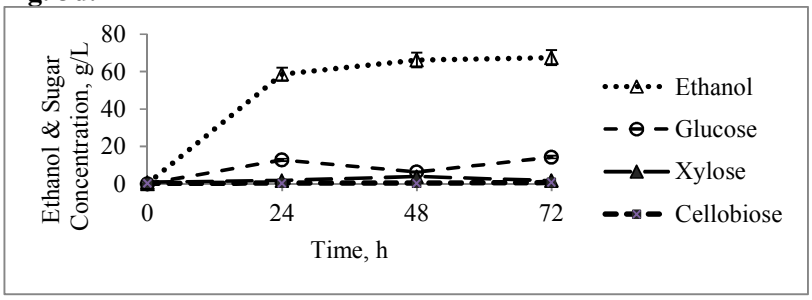


Fig. 3e.

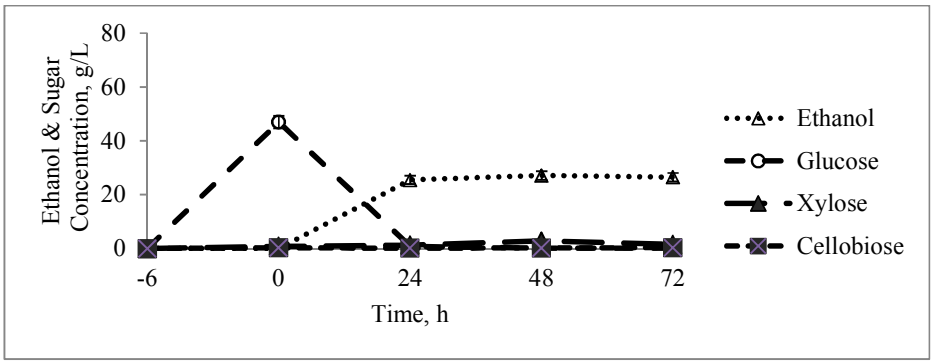


Fig. 4a.

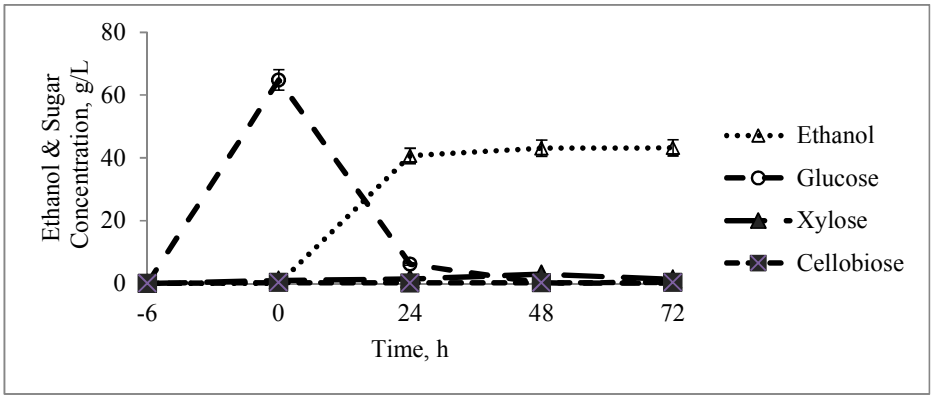


Fig. 4b.

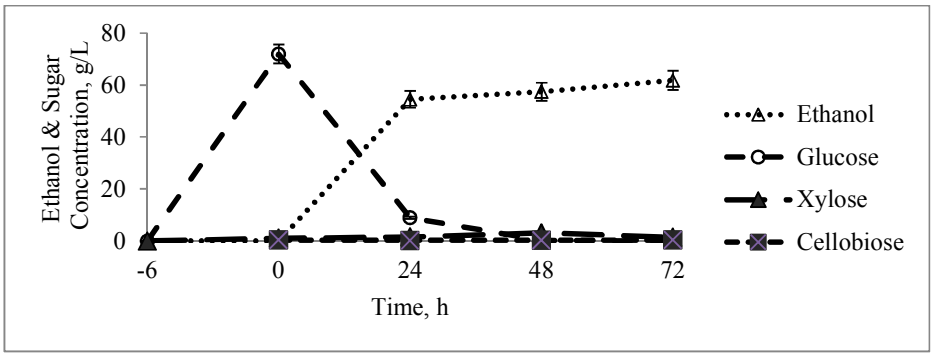


Fig. 4c.

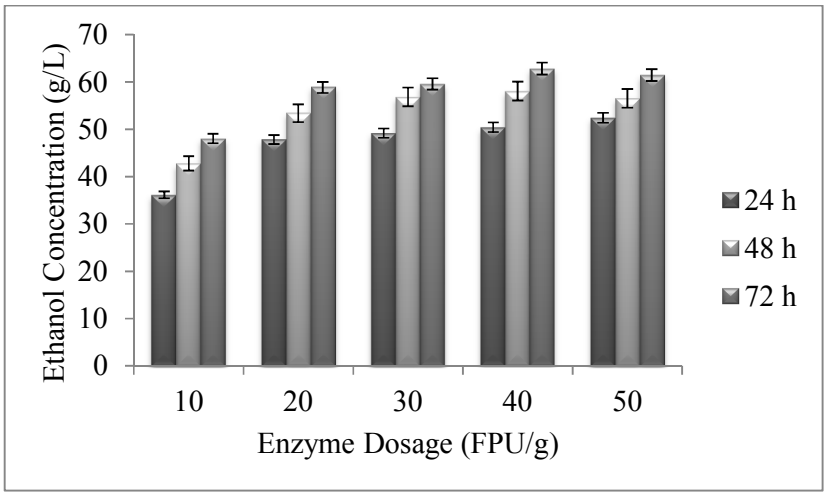


Fig. 5.