SUMMARY AND CONCLUSIONS

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Isolation and characterization of thermo and inhibitor tolerant yeasts

A total of 150 thermotolerant yeasts were isolated from various fruits, fruit juices, bagasse, and soil samples and waste samples collected from sugar mill and distilleries. Two most potential ethanol fermenting thermotolerant yeast were identified as *Kluyveromyces marxianus* JKH5 and *Pichia kudriavzevii* JKH1 based on their ITS-5.8s rDNA sequence analysis. These two potential yeasts were superior to rest of the isolated yeasts and standard yeast cultures due to their capability to produce ethanol titer of 55 and 54 g/L, respectively, within 18 h incubation at 42°C.

Enhancing tolerance of potential yeast strain(s) against temperature and inhibitors stress through adaptive laboratory evolution

The selected strains *K. marxianus* JKH5 and *P. kudriavzevii* JKH1 had low tolerance to inhibitors and tolerated only 3.5 g/L acetic acid, 2 g/L furfural, 2 g/L vanillin individually, and 2+0.2+0.2 g/L, respectively, of the same inhibitors mixed together in the inhibitor cocktail. Therefore, the strains were further improved to tolerate higher inhibitor concentrations through adaptive laboratory evolution (ALE) by growing them continuously in the presence of gradually increasing levels of inhibitors. After improvement, the strains were able to tolerate 6 g/L acetic acid, 3.2 g/L furfural, 3 g/L vanillin, individually, and 3+1+1 g/L, respectively, of the inhibitors present in the cocktail together. The adapted yeast *K. marxianus* JKH5 C60 had 60% and 80% improved ethanol productivity while fermenting glucose with initial concentration of 50 and 100 g/L, respectively, compared to the performance of its parent strain. In presence of inhibitors, adapted strain had shorter doubling time with reduced lag period and better specific growth rate.

Optimization of bio-process for cellulosic ethanol production by adapted yeast at shake-flask level

The method for sequential dilute acid-alkali pretreatment of sugarcane bagasse was optimized, using dilute sulfuric acid (3%, v/v) and dilute sodium hydroxide (5%, w/v) to overcome the biomass recalcitrance and improve the enzymatic hydrolysis of the lignocellulosic biomass. Upon optimized sequential dilute acid alkali pretreatment of sugarcane bagasse, the cellulose content increased from 51% to 83% and the hemicellulose content decreased from 23 to 1.4% together with a delignification of 97.2%, compared to native sugarcane bagasse. The pretreated biomass was characterized by various biophysical techniques, such as X-Ray diffraction (XRD), Scanning electron microscopy (SEM), Fourier transforming infra-red (FT-IR) spectroscopy, Thermo-gravimetric analysis (TGA) and Small angle neutron scattering (SANS) analysis. The increased cell wall porosity of the pretreated biomass, a favorable factor for improved enzymatic hydrolysis, was confirmed by increase in number of small pores and decreasing in number of large pores as analyzed by small angle neutron scattering. This is the first report on the successful application of SANS for unravelling and monitoring ultra-structural changes during deconstruction of sugarcane bagasse.

The hydrolysis of pretreated sugarcane bagasse resulted in enhanced titre of fermentable sugars at 125 g/L (78% saccharification) due to increased accessibility of biomass to cellulase. Separate hydrolysis and fermentation (SHF) of sequentially pretreated biomass for bioethanol production was performed by employing the adapted strain *K. marxianus* JKH5 C60, resulting in production of ethanol titer of 54 g/L. The same yeast was also employed for simultaneous saccharification and fermentation (SSF) of sequentially pretreated bagasse (at a solid loading of 15%, dry wt.) for enhanced production of bioethanol in presence of inhibitor cocktail. SSF by the adapted yeast resulted in an ethanol titer of 58.5 and 54.8 g/L ethanol,

when fermentation was performed in the absence (control) and presence of inhibitor cocktail, respectively.

Improvisation and scale-up of bio-process for high gravity simultaneous saccharification and fermentation of sugarcane bagasse at bench-scale fermenter

Batch-SSF of pretreated biomass (at a solid loading of 15%, dry wt.) for ethanol production using adapted yeast was successfully scaled-up to 3L lab-scale fermenter of SCB, resulting in enhanced production of ethanol with titers of 67.2 and 64.8 g/L, respectively, in absence (control) and presence of inhibitor cocktail. High solid loadings (18, 20 and 30%, dry wt.) of the pretreated biomass were also evaluated for enhancing the ethanol titer, yield and productivity. But the yield and efficiency of the ethanol production declined drastically from 80% (15 % solid loading during control experiment) to ~40% (30% solid loading) with rise in solid loading. Therefore, fed-batch strategy for enhanced ethanol production bagasse at higher solid loadings was adopted with different feeding strategies, employing feeding of biomass alone or feeding of biomass along with enzyme. The latter approach proved better for enhanced ethanol production at high gravity of biomass under shake-flask and was successfully employed for further scale-up at fermenter level. High gravity Fed-bath SSF of sequentially pretreated sugarcane bagasse (with intermittent feeding of biomass and enzyme and having a final solid loading of 20 %, dry wt.) produced 84.9 g/L ethanol with a productivity of 3.5 g/L/h under shake-flask. Under similar conditions, but in presence of inhibitor cocktail, fed-batch SSF of sequentially pretreated bagasse resulted in an ethanol titer of at 73.4 g/L under lab-scale fermenter.

Pentose sugars, majorly xylose, resulting from the dilute-acid pretreatment of sugarcane bagasse were also employed for ethanol production using pentose fermenting yeast. Before fermentation, the acid-hydrolysate was over-limed and detoxified to remove more than ~80%

inhibitors. Fermentation of the detoxified acid hydrolysate by *Pichia stipitis* NCIM 3499 resulted in production of ethanol with a titer of 6.8 g/L.

Mass balance analysis of the whole bioprocess for conversion of sugarcane bagasse to bioethanol using the adapted yeast strain was also performed to assess overall bioconversion. It indicated that overall, 260.1 kg of bioethanol could be produced per tonne of native sugarcane bagasse.

CONCLUSIONS

This study successfully developed a new robust thermo- and inhibitor tolerant yeast *Kluyveromyces marxianus* JKH5 C60 via adaptive laboratory evolution to tolerate higher concentrations of inhibitory compounds which are generated during pretreatment of biomass. Furthermore, the process of sequential dilute acid alkali pretreatment of sugarcane bagasse was developed to improve its enzymatic digestibility. Sequential pretreatment method could be employed for pretreatment of other lignocellulosic biomass also for enhancing the biomass conversion for biorefinery applications. Batch and fed-batch process of simultaneous saccharification and fermentation of pretreated bagasse were also optimized at shake flask and successfully scaled-up to 3L lab-scale fermenter using the adapted yeast. Comparison of the batch and fed-batch SSF of biomass revealed that fed-batch SSF was a better strategy for producing higher titers of cellulosic ethanol under high-gravity conditions. Pentose sugars retrieved from the dilute acid pretreatment step were also fermented successfully under biorefinery approach, to enhance overall yield of ethanol. Overall results this study indicate the developed yeast can help decrease the overall cost, time, and wastewater generation during high titer bioethanol production by eliminating the need to wash the pretreated biomass prior to fermentation.

Major limitation of the present study was that the fermentation of the unwashed pretreated biomass in the presence of actual acid hydrolysate having inhibitory compounds could not be optimized. Therefore, future studies should consider employing the developed strains for cellulosic ethanol production from unwashed biomass or pretreated biomass slurry (without liquid separation) obtained after dilute acid pretreatment of sugarcane bagasse. Furthermore, the adapted yeast strains can be evolved to ferment xylose present in the acid hydrolysate. The optimized process of enhanced bioethanol production from sequentially pretreated

sugarcane bagasse via fed-batch SSF can be further scaled-up to pilot scale fermenter in future.