International Journal of Modern Science and Technology, 2019;4(5):128-137.
ISSN: 2456-0235.
www.ijmst.co

Research Article

A Novel Strain Developed through Protoplast Fusion for Consolidated Bioprocessing of Lignocellulosic Waste Mixture

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Abstract

A consolidated bioprocessing (CBP) strain is capable of producing cellulolytic enzymes to saccharify the lignocellulosic biomass and fermenting the produced monosaccharides to ethanol. In this work, protoplasts of *Trichoderma harzanium ATCC® 20846™* and *Saccharomyces cereviseae* were obtained and they were fused using a PEG fusion method. The fused protoplasts were regenerated and screened for their cellulolytic activity and fermentative activity. An optimized Consolidated Bioprocessing was carried out for 160 h using the fused protoplasts showing a regeneration frequency of 5 x 10^{-4}. Around 0.04 g/L ethanol was produced by the CBP strain with a percentage cellulose conversion of 1.96%. SDS PAGE analysis was done to observe the proteins secreted by the CBP strain. The enzyme activities of the produced cellulase observed at the end of 160 h were FPases: 0.9 IU/mL, CMCases: 6.30 IU/mL, Xylanases: 352.9 IU/mL and Beta-glucosidases 1491.2 IU/mL.

Keywords: Protoplast fusion; Consolidated bioprocessing; Lignocellulosic waste; Regeneration frequency; Cellulase.

Introduction

The biorefinery concept of employing waste lignocellulosic biomass as a source for the second generation biofuel production is due to its easy availability, inexpensiveness, and the environment friendly green approach used for production. Bio-ethanol fuel blends are a vogue now in flex fuel vehicles and also as a stand-alone fuel in modified automobile engines Consolidated Bioprocessing (CBP), a process which integrates all the biorefinery steps of biomass pre-treatment, cellulase production, saccharification and ethanol fermentation, is a novel approach which is in its nascent stages [1].

A single genetically engineered microbial strain or consortia of strains with close cultivable temperatures are used in the process of CBP [1]. No external enzyme is required to be added [1-3]. Genetic engineering to produce a cellulolytic yeast, metabolic engineering of the pathways of cellulolytic organisms to produce ethanol by fermentation, a consortia of cellulolytic and fermentative organisms, protoplast fusion of cellulolytic and fermentative microbes, enhancing the natural CBP potential of certain *Clostridium* strains are some of the widely used approaches for Consolidated Bio-Processing of Lignocellulosic Biomass to produce ethanol [1-9].

One of the comparatively inexpensive methods of intergenic/intragenic hybridization of two specie to produce heterokaryons which later become stable to produce a single hybrid strain is the process of protoplast fusion [5-9] the process involves the isolation of protoplasts from the two participating microbial strains. Protoplasts are the spores devoid of the cell wall. Protoplasts are obtained using certain cell wall digesting enzymes procured commercially or produced in-house. The generated protoplasts are fused by several methods such as spontaneous, induced, mechanical, electrofusion, chemical. The molecular distance between the two protoplasts should be within 10 A. the CBP strain doesn't suffer feedback inhibition by the produced glucose in a saccharification broth as the glucoses are rapidly fermented to ethanol by the fermentative activity of the CBP strain [10-14].

In the present work, it was hypothesized that a CBP strain developed using protoplast fusion of *Saccharomyces cereviseae* and *Trichoderma harzanium ATCC® 20846™*

Received: 18.04.2019; Received after Revision: 06.05.2019; Accepted: 06.05.2019; Published: 08.05.2019
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protoplasts would directly convert the biomass to ethanol.

Materials and methods

Materials

The reagents used in the below mentioned sub-headings were laboratory grade reagents procured from M/s HiMedia Laboratory Pvt. Ltd., Mumbai, India.

Composition of Vogel's minimal media

The composition of Vogel's media is as follows: Tryptone (1 g/L), Tri-sodium citrate (2.5 g/L), Di-Potassium hydrogen phosphate (5 g/L), Ammonium nitrate (2 g/L), Magnesium sulphate heptahydrate (1.4 g/L), Calcium chloride dihydrate 0.1g/L, Tween 80 – 0.2 % (v/v). A Trace element solution containing Citric acid monohydrate 5g/L, Zinc sulphate heptahydrate 5 g/L; ferrous ammonium sulphate 1 g/L, Copper sulphate 250 mg/L, Manganese sulphate 50 mg/L ; Boric acid 50mg/L, Sodium molybdate 50 mg/L was prepared. 1 mL of trace elements per litre of the total media components was added [15]. The pH was set at 5.5 before autoclaving.

2.2 Isolation and fusion of protoplasts

Primary culture for protoplast isolation

Three day old culture of *Saccharomyces cereviseae* and 5 day old cultures of *Trichoderma harzanium ATCC® 20846™* from PDA slants were individually inoculated in Erlenmeyer flasks having autoclaved potato dextrose broth (100 ml) and incubated in a shaking incubator at 30 degree C, 3 days and 28 °C, 5 days respectively [11] . The mycelia of *Trichoderma harzanium ATCC® 20846™* were separated by filtration using an autoclaved cheese cloth to remove the mycelia debris. It was later washed with double distilled water. Yeast cells were collected by centrifugation at 5000 rpm for 20 min at 4 °C. The supernatant was discarded. The pellets were washed with double distilled water [6-9,16].

Enzyme mixture for protoplast generation

The 3 mL protoplast generation enzyme consortia contained (1:1:1) ratios of chitinase, pectinase and concentrated cellulases (obtained from *A. niger* with an enzyme activity of 10 IU/mL FPU). The osmotic stabilizer contained 0.6M Potassium chloride prepared in 0.1 M phosphate buffer and the pH was maintained at 6. The water used for the preparation was autoclaved double distilled water [6-9,16].

Production of protoplasts

Similar procedure was followed for the generation of protoplasts from *Saccharomyces ceresviseae* and *Trichoderma harzanium ATCC® 20846™*. The mycelia and the separated yeast cells were taken in separate vials and to the individual vials were added 1 mL of the osmotic stabilizer solution and 3 mL of the protoplast generation enzyme consortia. The mixtures were individually incubated at room temperature (28-30 °C) for 3 h. The mixtures were filtered using a double layered muslin cloth and washed well using the osmotic stabilizer solution to free them from the hyphal debris. A step of centrifugation was performed to the filtrate obtained at 5000 rpm for 15 min at 4 °C followed by washing using the osmotic stabilizer. The pellets were then suspended in the osmotic stabilizer solution and maintained at a temperature a little below the room temperature. The generation of the protoplasts were confirmed under the microscope at 100X and oil immersion. The cell wall was not present around the spores and yeast cells. The yield of protoplasts was measured using a haemocytometer [6-9,16].

Regeneration of protoplasts

The protoplasts were regenerated on PDA plates. Appropriately diluted (using osmotic stabilizer) protoplasts were spread plated on PDA plates and grown at appropriate temperatures for the growth of *Saccharomyces cereviseae* and *Trichoderma harzanium ATCC® 20846™*. The regeneration was also assessed by cultivation in PDB and assessment of the appearance of the protoplasts under a microscope [6-9,16]. The regeneration frequencies of the individual protoplasts were calculated using Eq. (1) [16].

$$\text{Regeneration Frequency} = \left(\frac{\text{No.of protoplasts regenerated}}{\text{Total no.of protoplasts plated}}\right) \quad (1)$$

Fusion of the protoplasts

The PEG-fusion buffer contained Polyethylene Glycol Mol.Wt. 4000 30% (v/v) prepared in 0.05 M Calcium chloride.dihydrate, and glycine 0.05 M at a pH of 7.5. (5:1) ratio of *Trichoderma harzanium ATCC® 20846™*:

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Saccharomyces cereviseae protoplasts (based on protoplast count) were chosen and an equal volume of PEG-fusion solution was added (1:1) of (Protoplasts : fusion solution). The mixture was shaken at fixed intervals and allowed to stand for 10-15 min at room temperature. An equal volume of the osmotic stabilizer solution was added to the fusion mixture and a small portion of it was visualized under the microscope at 100X oil immersion to visualized fused protoplasts [6-12,14,16].

Screening and selection of the fusion products

The fused protoplasts were plated in duplicates on 1% (w/v) microcrystalline cellulose containing Vogel's minimal agar plates. After 3-5 days of incubation, one of the duplicate plates were screened using the Gram's Iodine plate assay to visualize the production of cellulases. The fusant grew like *Saccharomyces cereviseae* but possessed the cellulase secreting property of *Trichoderma harzanium ATCC® 20846™* [16].

Gram's iodine plate assay

Gram's Iodine was flooded on the microcrystalline cellulose agar plates with Vogel's minimal media and after 3-5 min was washed with distilled water. Iodine bound to the region containing cellulose while the regions having no cellulose due to cellulose hydrolysis by cellulases showed a clear zone. This screening procedure was used to zero in on functional fusants to assess the quality of the intergenic fusion [16,17].

A novel substrate mixture

A cost efficient and novel cellulose substrate was chosen for the production of cellulases. Surgical absorbent and non-absorbent cotton, cotton gauze, are used aplenty by the hospitals and clinics on a day-to-day basis [18]. These are considered as biological wastes and are discarded by the hospitals after a customary practice of disinfecting them chemically or by steam sterilization methods [18]. Waste cottons from the non-infectious, non-pathological division were collected. Cottons are known for their high percentage of celluloses [19]. Packaging card boards which are high in cellulose content were also chosen [20]. Supermarkets discard a lot of packaging material every day when they are damaged and fungal-infested making them unsuitable for recycling purposes. In an attempt to using the cheapest cellulose substrate for the production of cellulases, we collected these waste materials. This would also decrease the generation and accumulation of waste. The usage of waste material for fuel production would be another added advantage of this choice.

Preparation of the novel cellulosic substrate mixture

The disinfected surgical waste cotton was subjected to a stage of autoclaving prior to washing them using a mixture of disinfectants. Proper laboratory personnel protective equipments were used for the purpose. Once the cotton was processed using the above approaches, it was dried in a hot air oven at 30 °C until it was completely dry. Damaged waste packaging card boards were washed with water repeatedly to get rid of visible dirt. The card boards were shredded to uneven sizes in a cardboard shredder (0.5 cm average size). After shredding, it was finely powdered using a mixer grinder. The dried cotton was pulverized to a fine powder though some of the strands remained intact. The card board and cotton mixture were used in a 1:1 ratio for the submerged fermentation process for the production of cellulases.

Optimization of consolidated bioprocessing

The process of submerged fermentation of the CBP strain was optimized for the inoculum concentration (v/v) % and the number of days of incubation. The rest of the conditions to carry out submerged fermentation were the same as that mentioned consolidated bioprocessing of the cellulosic mixture.

Consolidated Bioprocessing of the Cellulosic Mixture

The submerged batch fermentation of 1 Litre culture volume was carried out in a 3.2 L Bioengineering KLF Advanced Bioreactor with automated controllers. The minimal media composition was the Vogel's Minimal media mentioned above with 1% (w/v) of the 1:1 ratio of finely powdered surgical waste cotton and cardboard mixture.

Primary inoculum

The primary inoculum was prepared in autoclaved 150 mL Vogel's media in an Erlen Meyer flask containing 1% surgical waste cotton

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and waste card board mixture. A spore suspension containing ($3x10^9$ /mL) spores was used to prepare the starter culture. It was incubated in a rotary incubator at 28 °C for 5 days prior to inoculation.

CBP in the bioreactor

A working volume of 1 litre (media + inoculum) was to be used in the bioreactor. 850 mL of Vogel's media containing 1% surgical waste cotton-card board mixture (1:1 ratio) was used as the media. 2 N sodium hydroxide and 2N hydrochloric acid were used to maintain the pH. 5-10 ppm of polypropylene glycol was used as the antifoam. 15% (v/v, %) inocula from the starter culture was used to inoculate the fermentor aseptically. The fermentor was set in the automatic mode with a set point of 5.5 for pH and a set temperature of 28 °C. Aeration was varied manually from time to time between 1 vvm to 0.5 vvm based on the foaming. Agitation and the DO% were cascaded. The high point of agitation was 160 rpm while the low set point was 100 rpm. DO% high set point was 80% and lowest was 60%. An anchor type impeller was used keeping in mind the broth's viscosity increase after 36 h.

Enzyme and ethanol harvest

Sampling was done at an interval of 24 h to estimate the enzyme activity. After the maximum enzyme activity was estimated at the end of 7 days, the fermentor was stopped and the broth was harvested through the bottom harvest port. The broth was centrifuged at 6000 rpm at 4 °C for 20 min. The supernatant was assessed for enzyme activity using the standard IUPAC DNSA method [21] while the produced ethanol was quantified using a HPLC. The percentage cellulose conversion to ethanol was estimated using the standard NREL Eq. (2).

$$\% \text{ Cellulose Conversion} = \left(\frac{(Ethanol)f-(Ethanol)i}{\{0.51\times(f\times Biomass)\times 1.111\}}\right)\times 100 \quad (2)$$

Where,
(Ethanol) f – Final Concentration of ethanol after fermentation (g/L).
(Ethanol) I – Ethanol initial concentration in broth (g/L).
Biomass – Dry biomass (g/L) at the beginning of fermentation.
f – Fraction of dry biomass containing cellulose (g/g)
0.51 – Conversion factor for the conversion of glucose to ethanol based on stoichiometric biochemistry of yeast.
1.111 – converts cellulose to glucose equivalent.

Dry biomass weight measurement

The dry biomass weight was measured using a slightly modified method of Aftab and Patrick 2008 [22]. The residual substrate concentration was also measured in this method. The total protein content from time to time was measured using the Lowry's method.

HPLC analysis

A HPLC analysis was performed using an Agilent 1290 Infinity HPLC with a Refractive Index detector. The mobile phase was sulfuric acid 0.005 M. 20 microlitre of the samples were injected. The oven temperature was 60 °C and the detector temperature was 55 °C. The column was Agilent Hi-Plex H 7.7 mm x 300 mm x 8 mm.

SDS PAGE analysis

SDS PAGE analysis using a 10% separating gel was carried out for the extracellular proteins of *Trichoderma harzanium ATCC® 20846™*, *Saccharomyces cereviseae* and the CBP strain. The molecular weight of the proteins produced by the CBP intergenic fusant would assure that the fusants are capable of cellulolytic and fermentative activities.

Results and discussion

Isolation and fusion of protoplasts

In fig. 1, the white region shows that iodine has not bound to the regions lacking cellulose which have been consumed by the cellulases secreted by the growing protoplasts [17]. In the process of protoplast fusion, the molecular weight of the PEG used, the pH of the fusion buffer, the temperature, time of incubation and the concentration and strength of Calcium chloride played a major role in initiating the fusion [7,9,16]. When PEG fusion buffer was added to the protoplasts, they came closer and were observed as clusters in pairs following which a dissolution of the plasma membrane of each of the protoplasts resulted in coming together of the protoplasm of the two strains [7,23] the exact mechanism of protoplast fusion is still not understood entirely. However, when protoplasts are in close vicinity, the fusion agents result in the alteration of the lipid and

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protein content of the protoplasts enabling the walls of the participating protoplasts to come into proximity. The Ca^{2+} of the Calcium chloride enables an alteration in the zeta potential of the plasma membrane of the protoplasts thus enabling the high molecular weight negatively charged PEG making a contact and leading to fusion of the protoplasts consequentially. The age of the culture from which the protoplasts are to be generated played a major role. The lesser the age of the culture the higher was the protoplast's production rate [16]. Older culture would be more tolerant to the stresses induced by the cell wall lytic enzymes. Some researchers such as Couteaudier, Viaud and Riba 1996 [24] have performed genetic analysis of the protoplast fusion products and concluded that protoplast fusion, the asexual alternative to genetic recombination has great potential to generate intergenic hybrids.

The heterokaryons/heterozygotes that resulted out of the intergenic protoplast fusion were screened using specific methods [6,16] described below [17]. The fusants began to appear within 48 h of plating the fusion products. Careful and repeated re-platings of the selected fusants on microcrystalline agar plates were done to eliminate the minor contaminations by the pure culture of *Trichoderma harzanium ATCC® 20846™* and *S.cereviseae* and obtain stable intergenic hybrids capable of regeneration [16]. A control test made on the microcrystalline plates revealed that the pure protoplasts of the either strains which did not fuse would not give positive results on the cellulolytic and fermentative assays [24]. Microscopic examination revealed that though initially the intergenic fusion products were bi-nucleate (Fig. 2), in the course of repeated subculturing the stable fusants became uninuclear showing high mitotic recombination possible by protoplast fusion. Such an uninucleate fusant elucidates the probability that *S.cereviseae* and *Trichoderma harzanium ATCC® 20846™* are compatible for somatic hybridization at the molecular level [6]. Reports also suggest that strains without compatibility to live together at the molecular level would separate quickly resulting in unstable fusants that die out during repeated subculturing [16,23].

During the intergenic hybrid fusants' growth on solid media it was observed at times that the CBP strain did not resemble *S.cereviseae* morphology but possessed fusant characteristics. This could be because the nuclear fusion did not occur and just the cytoplasm of the two strains has fused. It is because of this reason that protoplast fusion cannot strongly vouch for a stable heterokaryotic intergenic fusion product. In some cases of subculturing it was observed that the fusants gave rise to parent strains without the fusant characteristics.

The regeneration frequencies of the protoplasts were estimated during each stage of individual protoplast regeneration and protoplast fusants regeneration. The Regeneration frequencies were thus: *Trichoderma harzanium ATCC® 20846™* protoplasts: 6×10^{-4}, *Saccharomyces cereviseae* protoplasts : 7.5×10^{-3}, Fused protoplasts: 1.6×10^{-4}, Fused protoplasts after 160 hours submerged fermentation : 5×10^{-4}. The regenerated fusants were approximately 10^3- 10^4 times lesser than the population actually plated [6,8]. This could be attributed to the fact that some fusants were reversing to the parental strains or were not able to co-exist in the intergenic environment [16].

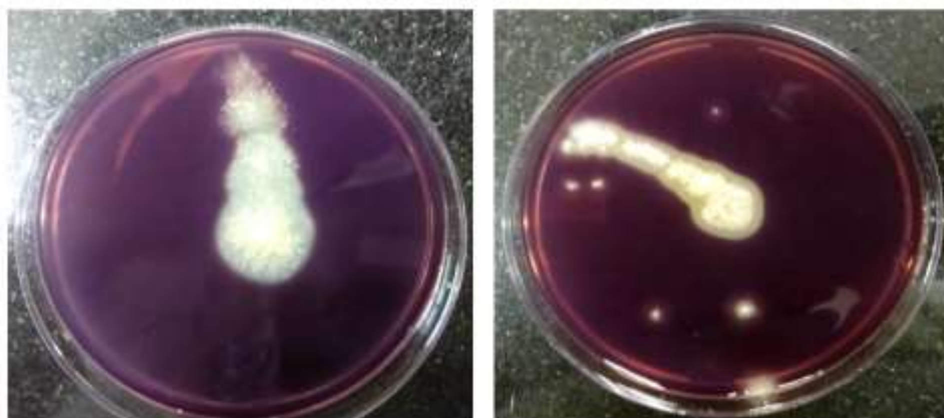

Fig. 1. Gram's Iodine Plate Assay to screen the cellulolytic activity of the CBP strain developed through protoplast fusion

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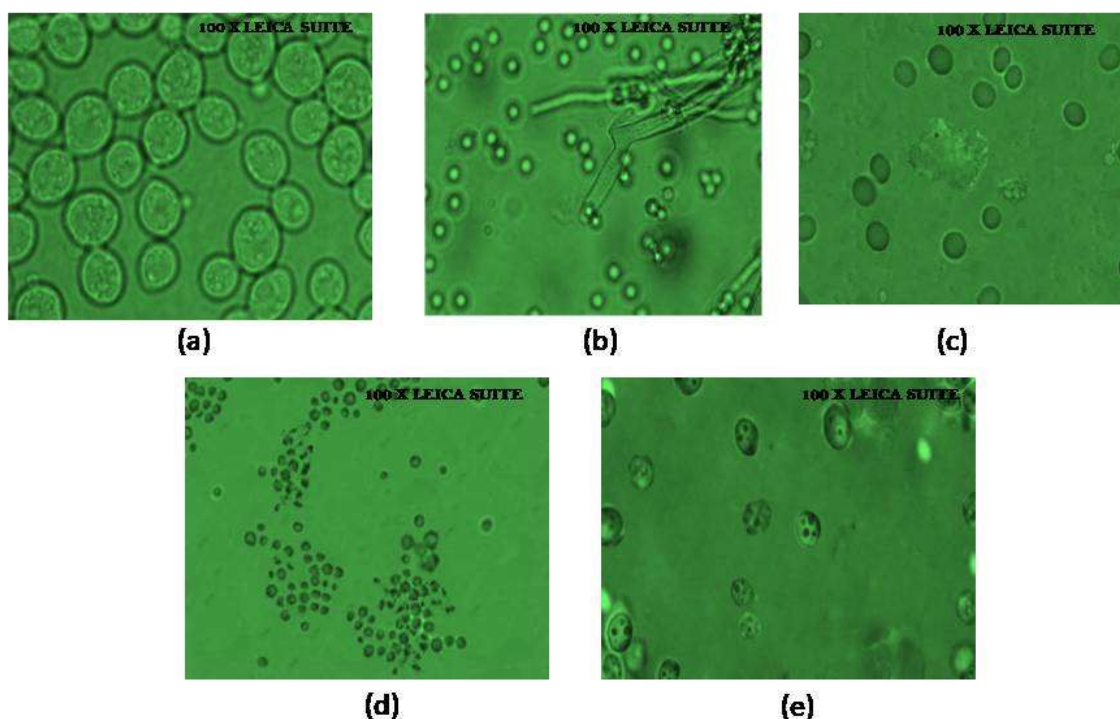

Fig. 2. (a) *Saccharomyces cereviseae* cells under the microscope, (b) spores of *Trichoderma harzanium ATCC® 20846™* releasing from the hyphae (c) Yeast protoplasts lacking the cell wall (d) Isolated protoplasts of *Trichoderma harzanium ATCC® 20846™* and (d) Fused protoplasts of *Saccharomyes cereviseae* and *Trichoderma harzanium ATCC® 20846™*

SDS PAGE analysis of the fusant's extracellular protein content

The SDS PAGE analysis of the extracellular proteins from the broth were run in separate lanes. *Trichoderma harzanium's* extracellular proteins showed the presence of Exoglucanases at molecular weights 47.2 KDa and 52 KDa, Endoglucanases at molecular weights of 42 KDa, 33 KDa, 22 KDa and 25 KDa, Xylanases at the molecular weight of 29.8 KDa and Beta-glucosidasea at the molecular weights of 23 KDa, 75 KDa and 52 KDa. The SDS PAGE analysis is shown in fig. 3. The extracellular proteins of *Saccharomyces cereviseae* showed bands at various molecular weights which do not correspond to the cellulases molecular weight which was reconfirmed using an Gram's Iodine Plate Assay of pure *Saccharomyces cereviseae* culture on Micro Crystalline Cellulose Plates [17].

Lane 4 was loaded with the total extracellular proteins of the CBP strain. The last lane showed proteins corresponding to the molecular weights of Cellulases and also contained proteins which were characteristic of the *Saccharomyces cereviseae* total protein content. The results of the gel clearly assure that the CBP strain secretes cellulases and possesses the properties of *Saccharomyces cereviseae.* As such there are no unique methods to screen the intergenic fusants in a protoplast fusion procedure [25]. Methods suitable to the application of the fusants have been used worldwide.

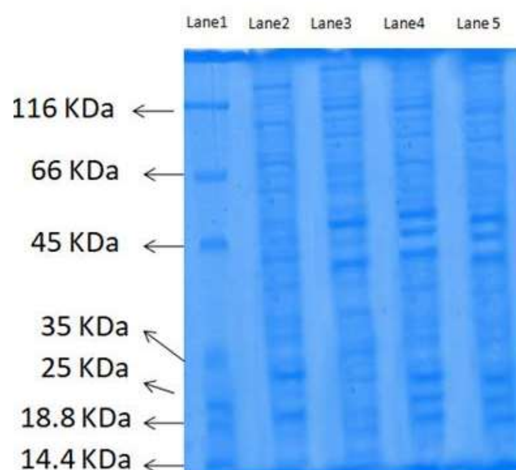

Fig. 3. SDS PAGE gel. Lane 1: molecular weight marker. Lane 2: Extracellular proteins of *Trichoderma harzanium ATCC® 20846™*, Lane 3: Extracellular proteins of *Saccharomyces*

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cereviseae. Lane 4: Extracellular proteins of the CBP strain and Lane 5: Concentrated extracellular proteins of the CBP strain

Optimization of consolidated bioprocessing

The CBP strain was optimized for submerged fermentation using the surgical waste cotton and waste cardboard mixture as the cellulosic substrate. In the process of optimization, three time of incubation: 5 days, 7 days and 9 days and three inoculum ratios (v/v)%: 10, 15 and 20% were used. The optimum condition was estimated based on the maximum possible ethanol production.

When 10, 15 and 20% (v/v) of inoculum were used for 5 days of incubation no ethanol was produced. When 10% (v/v) inoculum was used for 7 and 9 days around 0.02 g/L ethanol was produced which proved that increasing the number of days of incubation proved to yield higher ethanol content.

When 15% (v/v) inoculum was used for 7 days and 9 days, 0.04 g/L ethanol was produced. Similarly, when 20% (v/v) inoculum was used for 7 days and 9 days, around 0.04 g/L ethanol was produced. The above results let us conclude that a 7 day incubation period and 15% (v/v) inoculum was the optimum condition for the production of ethanol. Optimization is expressed as a response surface curve as shown in fig. 4.

Consolidated bioprocessing of the novel cellulosic mixture

The enzyme activities obtained using the batch SMF process at the end of 160 h were FPases: 0.9 IU/mL, CMCases: 6.30 IU/mL, Xylanases: 352.9 IU/mL and Beta-glucosidases 1491.2 IU/mL. The starter culture of CBP in the Vogel's medium contained a significant amount of cellulases which may have functioned as an inducer as well [26]. Agitation with a low shear rate did not cause considerable breakage of the growing culture [27]. Addition of excessive antifoam decreases foam and hence influences negatively the KLa, the mass transfer coefficient. A serious issue of catabolite repression was observed in the fermentation. Cellobioses themselves inhibit and cause catabolite repression during cellulase production [28]. A FPase activity of 0.9 IU/mL is the maximum activity obtained at the end of 160 h. This catabolite repression also results in no further usage of cellulose in the media resulting in 2.5 g/L of substrate mixture being left as the residual cellulose in the fermentation media after 160 h. A high cAMP level in the cell needs to be maintained in order to facilitate the expression of more cellulases from the promoter. The glucose produced after 72 h were made use of by the CBP strain to ferment and produce ethanol. Though reports claim that the CBP strain would'nt / should'nt suffer catabolite repression/feedback inhibition by the produced glucoses [5] it did'nt apply to the strain produced in this work. The concentration of ethanol gradually kept increasing from miniscule quantities to 0.04 g/L ethanol (HPLC shown in fig. 5) at the end of 160 h with a %cellulose conversion of 1.96% which was lesser than the yield obtained using metabolically engineered/genetically manipulated yeast strains [2,4,13]. One major reason for choosing *Trichoderma harzanium ATCC® 20846™* and *Saccharomyces cereviseae* as candidates for protoplast fusion and CBP is their cultivable temperatures which vary slightly (28-30 °C). CBP using organisms of varying cultivable temperature would require a phase of adaptation prior to usage.

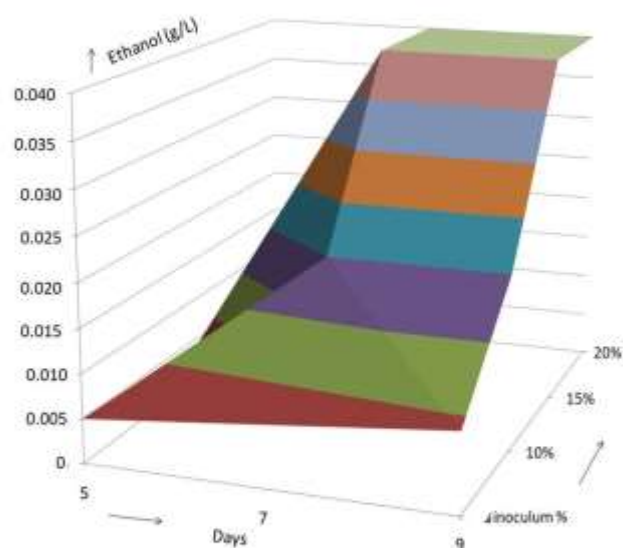

Fig. 4. Optimization of submerged fermentation of the CBP strain on the basis of ethanol production. 7 days of incubation

Growth characteristics of the CBP strain

In the submerged fermentation using the CBP strain, an Average Biomass yield coefficient $Y_{x/s}$ of 0.52 g of biomass/ g of substrate mixture was obtained. A maintanence coefficient of 0.02 g/g/h was obtained. A maximum growth rate of μ_{max} 0.06g/h was

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measured at 48-72 h (Tiina et al 2005). The maximum biomass yield was 8 g/L at the end of 160 h. After 160 h, though the cellulase production stalled, the biomass increase didn't halt [27] as the concentration of glucose and cellobioses helps the growth of the fungi. The pH decreased initially to a very low point of 3.2 pH in the fermentation. This indicated that the carbon source was being used up first along with the usage of the nitrogen source. A higher C/N ratio in the media components decreases the pH [26]. This liberates free H+ in the broth leading to a decrease in the fermentation pH. The DO% decreased to 20% during 36 to 72 h of growth. This was clearly in accordance with the observed increase in the biomass after 36 h [14]. The amount of ethanol produced by the CBP strain developed through protoplast fusion was 0.04 ± 0.02 g/L which is 4 x 10^{-4} g ethanol/ g dry biomass. The protoplast fusants generated from *S. cereviseae* and *Candida shehate* showed 0.424 g ethanol/g biomass as reported by Yan et al 2015 [29] reported that the protoplast fusants produced around 63.5 g/L ethanol in a 60 hour cultivation.

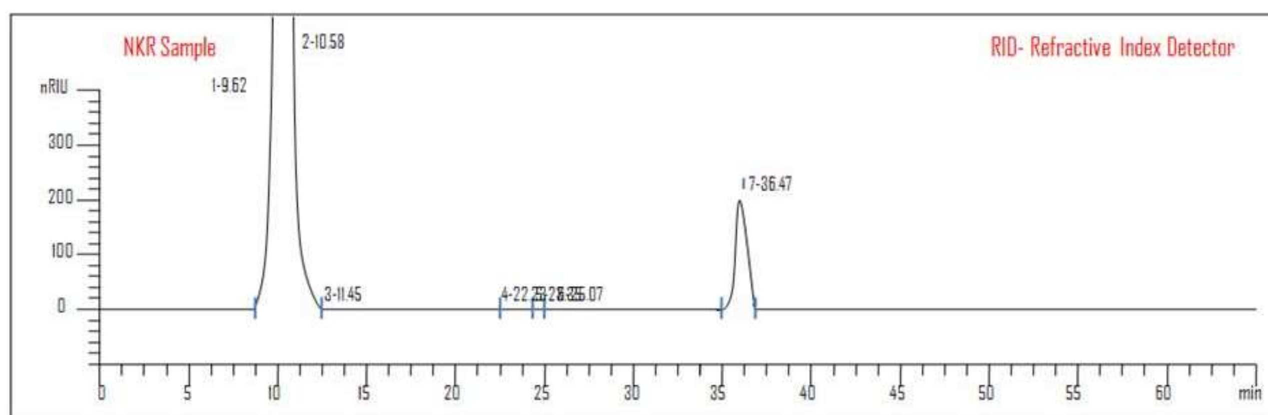

Fig. 5. HPLC chromatogram showing the presence of 0.04 g/L ethanol at a retention time of 36.47 min. 3g/L glucose was observed at a retention time of 9.62 min. 0.2 g/L Xylose and 0.04 g/L Arabinose were observed at Retention times of 10.6 and 11.4 min respectively

Conclusions

A Consolidated Bio-processing strain was developed by PEG mediated intergenic fusion of the protoplasts isolated from *Saccharomyces cereviseae* and *Trichoderma harzanium ATCC® 20846™*. A percentage cellulose conversion of 1.9% was observed using the CBP strain. Though the yield of ethanol was less using the CBP strain, future perspectives of fusing the protoplasts of much efficient strains may show potential towards higher yields. The CBP strain has the potential to reduce the cost of individual steps of the biorefinery approach of ethanol approach by 60-70% which could be an alternative to be used by the biofuel industries and researchers globally.

Conflicts of Interest

Authors declare no conflict of interest.

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