International Journal of Modern Science and Technology, 2018;3(5):117-125.
ISSN: 2456-0235.
www.ijmst.co

Research Article

Yeast Co-Culture with *Trichoderma harzanium* ATCC® 20846™ in Submerged Fermentation Enhances Cellulase Production from a Novel Mixture of Surgical Waste Cotton and Waste Card Board

S. Jason Charles[1], R. Navnit Kumar[2], T. R. Sambavi[2], S. Renganathan[2],*

[1]Molecular Biology Laboratory, Centre for Biotechnology, Anna University, Chennai. India.
[2],*Biofuels Laboratory, Centre for Biotechnology, Anna University, Chennai. India.

*Corresponding author's e-mail: rengsah@rediffmail.com

Abstract

Trichoderma harzanium ATCC® 20846™ when used in the process of submerged fermentation for the production of cellulases, suffers a catabolite repression due to the accumulation of glucose within the fermentation broth. A strain of yeast *Saccharomyces cereviseae* RW 143 was inoculated into the cellulase production fermentation containing *T. harzanium* ATCC® 20846™. In two different experiments yeast (5% w/v) whole cells and immobilized yeast cells (5%w/v) were used. The accumulated glucose was consumed by the yeast and *T. harzanium* ATCC® 20846™ was efficiently able to produce enzymes with increased activities. When no yeast cells were added, 1.946 FPU/mL was obtained at the end of 168 hr. When yeast whole cells were used 2.035 FPU/mL was obtained at the end of 96 hr and when calcium alginate-immobilized yeast cells were used 1.75 FPU/mL was obtained at the end of 120 hr. The 0.09 g/L ethanol was produced at the end of 96 hr when whole yeast cells were used for co-culture.

Keywords: *Trichoderma harzanium*; Cellulase production; *Saccharomyces cereviseae*; Co-culture.

Introduction

Trichoderma strains possess predominant asexual stage by producing asexual spores. A sexual phase is absent in most of the strains with a few exceptions under environmental stress which tend to later turn into the asexual ones. Cellulases play a significant role in industries such as the pulp and paper, textile, laundry, biofuel production, food and feed industry, brewing, and agriculture [1]. Fungal cellulases have a higher yield than bacterial cellulases. Filamentous fungi secrete the enzymes inside the media. Cellulases are a combination of three enzymes namely endoglucanases/CMCases (EC 3.2.1.4), exoglucanases/FPases, including cellobiohydrolases (CBHs) (EC 3.2.1.91), and β -glucosidase (BG) (EC 3.2.1.21). Cellulase uses these three enzymes to hydrolyze the beta1-4 glycosidic linkages in between the cellulose polysaccharide units to release the beta-D-glucose monomers.

Trichoderma harzanium is a filamentous asexually reproducing fungi with previously reported cellulolytic activities. It belongs to the Division Ascomycota and the family hypocreaceae [2]. In a phenomenon called Carbon Catabolite Repression CCR or catabolite repression, CRE I repressor activation takes place and the cellulase production is hampered or stopped in *T. harzanium* ATCC® 20846™ [3]. Such a phenomenon arises when there is excess of an easily available sugar instead of the tough to metabolize carbon source such as cellulose [3]. The circulating levels of cAMP intracellularly within *T. harzanium* ATCC® 20846™ needs to be high in order to facilitate the expression of more cellulases from the respective promoter region. A high cAMP level would be maintained only when a stress level arises due to the absence of easily metabolizable sugars [4].

Saccharomyces cereviseae metabolizes glucose to utilize in the biomass production and the production of ethanol following the fermentative pathway. The aerobic environment lets the yeast synthesize more biomass while the anaerobic environment results in the production of certain fermentative products such as ethanol, acetaldehyde. In a phenomenon called Crab tree

Received: 14.05.2018; Received after Revision: 27.05.2018; Accepted: 27.05.2018; Published: 28.05.2018

effect, even under highly aerobic conditions, yeast tends to produce ethanol [5]. The fermentative process in yeast utilizes the Embden Mayerhoff Parnas pathway. Appropriate sugar concentrations and aerobic / anaerobic environment will let the microbe accordingly metabolize the sugar [5].

Value added products are usually produced by a single microbial strain. The process of co-culturing involves the simultaneous aerobic / anaerobic cultivation of a group or microbial strains. However at the fundamental level growing two microbial strains with a particular degree of contact between them is co-culture [6]. Of late, co-cultures have been showing lot of promise compared to single micro-organism utilization. The capability of simultaneous usage of the metabolic pathways of the participating microbial strains contribute to some major bio-transformations in nature elucidating the natural and synthetic interaction among the specie involved [6]. In a co-culture, the productivity or growth of one of the participating microbial specei could be promoted or deterred by the activities of other micro-organisms present in the medium, the processing cost could become cheaper, formation of certain unique microbial metabolic products or by products could result. The biofuels and bioenergy sectors greatly rely on microbial co-cultures for processes such as Consolidated Bio-Processing and Simultaneous Saccahrification and Fermentation [7].

In the present work, to prevent Carbon Catabolite Repression in *T. harzanium* ATCC® 20846™ and produce more cellulases, a co-culture using *S. cereviseae* was performed. It was done with an approach that *S. cereviseae* would consume the glucose accumulated during cellulase production by *T. harzanium* ATCC® 20846™ and help in maintaining a higher secretion of cellulases by *T. harzanium* ATCC® 20846™.

Materials and methods

The strains were purchased from the ATCC, USA. The nutrient rich media Potato Dextrose Broth procured from M/s Hi-Media was used for the growth of the cellulolytic fungi. It was prepared according to the manufacturer's instructions and autoclaved at 121°C for 20 min. Other reagents used below were procured from the brand Hi-Media.

The composition of Vogel's media (g/L) 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), finely powdered surgical waste cotton-card board mixture in 1:1 ratio (1% w/v), Calcium chloride dihydrate 0.1 g/L, Tween 80 – 0.2 % (v/v). A Trace element solution containing Citric acid monohydrate 5 g/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 50 mg/L, Sodium molybdate 50 mg/L was prepared. 1 mL of trace elements per litre of the total media components was added. The pH was set at 5.5 before autoclaving.

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. 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. Waste cottons from the non-infectious, non-pathological division were collected [22]. Cottons are known for their high percentage of celluloses. Packaging card boards which are high in cellulose content were also chosen. 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.

Submerged Fermentation

The submerged batch fermentation of 1 Litre culture volume was carried out in a 3.2 L Bioengineering KLF Advanced Bioreactor with automated controllers.

Primary Inoculum

The primary inoculum was prepared in autoclaved 150 mL Vogel's media in an Erlen Meyer flask containing 1% Microcrystalline cellulose. 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. The primary culture for *S. cereviseae* was prepared by inoculating a loopful of the organism from YEPD agar plates. The liquid cultures were grown until 48 h in a conical flask. The broth was centrifuged and the pellets were used as the primary culture to initiate culture in w/v% of the broth.

Cultivation 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. 2N sodium hydroxide and 2N hydrochloric acid were used to maintain the pH. 5-10 ppm of polypropylene glycol was used as the antifoam. Prior to autoclave the pH probe was calibrated for two points of 7 pH and 4 pH. The media components along with the acid base and antifoam components were autoclaved at 121°C for 20 min. An external supply of cold water was done by connecting to a chiller running at 20°C to maintain the temperature. Once the media was cool and the component lines were connected, a constant agitation of 50 rpm was maintained and the DO probe was polarized with a constant supply of 1 vvm of air for around 6 hr. 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. Antifoam would automatically dispense based on the response by the foam sensor. Aeration was varied manually from time to time between 1 vvm to 0.5 vvm based on the foaming. Too much antifoam addition would be detrimental to the production of cellulases by hindering the mass transfer within the fermentor. 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 hr.

Enzyme Harvest and Enzyme Activity Estimation

Sampling was done at an interval of 24 h to estimate the enzyme activity. After the maximum enzyme activity was estimated, 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 contains the secreted enzymes. The enzyme activity was measured using the standard IUPAC Ghose method [8].

Dry Microbial Biomass Weight Measurement

The dry mycelial weight was measured using a slightly modified method of Ahmad et al (2008) [9]. The residual substrate concentration was also measured in this method. The fermentation continued until the substrate concentration reached constancy and no further substrate was being taken up and no increase in enzyme activity was observed. The total protein content from time to time was measured using the Lowry's method. The yeast biomass was homogenous in the broth and was easily pipetted out leaving behind the *T.harzanium* ATCC® 20846™ biomass along with unconsumed cellulose substrate.

Optimization of Yeast Co-Culture Process in Submerged Fermentation

Optimization of yeast co-culture to enhance cellulase production was carried out. 3, 5 and 7% (w/v) yeast was used for co-culture.

Yeast Co-Culture in the Submerged Fermentation

Optimization elucidated the best condition for achieving best cellulase yields. The three submerged fermentations to study the yeast

co-culture methods were performed separately. In a control fermentation, no yeast cells were added while in the other two, whole yeast cells and calcium alginate immobilized yeast cells were added separately. Whole yeast cells and yeast cells immobilized in calcium alginate beads were prepared separately. Cream of yeast grown until log phase in YPD media was inoculated (5% w/v) into the fermentation broth containing *T.harzanium* ATCC® 20846™ that produces cellulases. While using calcium alginate immobilized yeast cells, the volume of beads containing 5% (w/v) of yeast was inoculated into the *T.harzanium* ATCC® 20846™ containing bioreactor. Yeast cells grown to log phase were inoculated into the *T.harzanium* ATCC® 20846™ culture containing media which was 48 h old. The cellulase production media accumulates glucose after 48 h [3, 10, 12, 23-26]. Hence to consume the reducing sugars produced in the process, yeast was added.

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. Standards with known concentrations were run prior to analysis of unknown sample mixtures. 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.

Results and discussion

Optimization of Yeast Co-culture

Yeast co-culture was done using three different concentrations as shown in Fig. 1. 3% 5% and 7% (w/v) of yeast inocula were used for the process of co-culture. 3% showed very less enhancement of enzyme activity. 5% and 7% showed same levels of increase in the enzyme activity. Hence 5% (w/v) was chosen as the optimum yeast inoculum concentration for co-culture. A concentration lesser than 3% may not have any effect on the enzyme activity increase while a concentration more than 7% may become competitive for the growth of *T. harzanium* ATCC® 20846™ and also result in mass transfer limitations [13, 14] by contributing to the increase in the growth media's viscosity within the fermentor. Hence an optimum concentration of yeast inocula was chosen with an objective of preventing catabolite repression that reduces cellulase production and also to permit normal uninhibited growth of *T. harzanium* ATCC® 20846™, the cellulase producer. 3%, 5% and 7% (w/v) of yeast was co-cultured with *T. harzanium* ATCC® 20846™ to find out the required optimum concentration to enhance cellulase production. 5% (w/v) was found to be the optimum concentration that led to the yield of 1.95 FPU/mL activity.

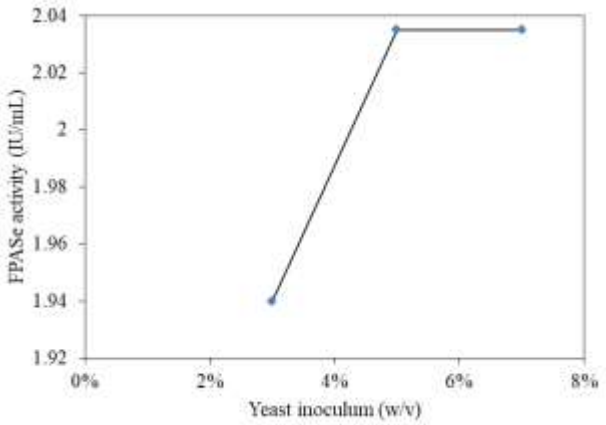

Fig. 1. Effect of inoculum on FPASe Activity

Yeast Co-Culture and Enhancement of Enzyme Activities

In a cellulase production batch process, after 48 h, the cellulases produced were sufficient to catalyze the conversion of the cellulose in the media to cellobioses and glucose [11, 12, 15]. The glucose produced may be consumed by *T. harzanium* ATCC® 20846™ and its necessity to synthesize cellulases reduces which may be the reason for the unconsumed 2g/L cellulose in the media [11-13]. The fermentation ended at around 160 h with an enzyme activity of 1.95 IU/mL FPU and 4.2 g/L glucose accumulation as seen in Fig. 2. High stress conditions and higher amount of cAMP would cause more production of cellulases. When glucose starts surfacing in the media, cellulase production would decrease owing to a phenomenon called catabolite repression [10-12]. To circumvent this issue and address the problem, yeast co-culturing was done. After 48 h of *T. harzanium* ATCC® 20846™ culture in the media, 5% yeast whole cells and 5% calcium alginate immobilized yeast cells were co-cultured in the media. When 5% (w/v) yeast whole cells were used for co-culturing, 1.2 g/L glucose accumulated at the end of 96 h as shown in Fig. 3 with an enzyme yield of 2.035 IU/mL FPU along with a minute fraction of ethanol of around 0.09 g/L ethanol as shown in the HPLC chromatogram given in Fig. 4. When

immobilized yeast cells were used 1.7 FPU/mL activity was recorded and no ethanol was produced in this process as shown in Fig. 5. The changes in the values of the enzyme activities for CMCase, Xylanases and Beta-glucosidases are given in Fig. 6.

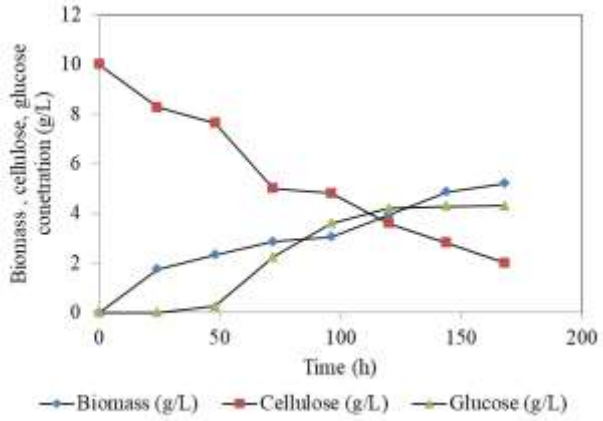

Fig. 2. Cellulase production by *T. harzanium* ATCC® 20846™ without co-culture

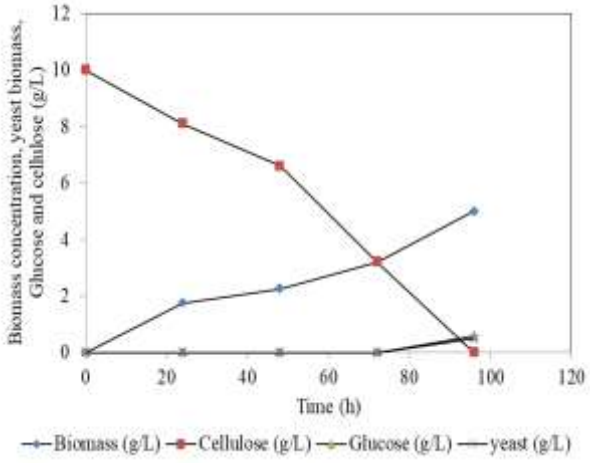

Fig. 3. Cellulase production by 5% (w/v) yeast co-culture with *T. harzanium* ATCC® 20846™

The growth parameters were analyzed. The Biomass Yield coefficients for *T. harzanium* ATCC® 20846™ Yx/s was 0.45 g biomass/g substrate without yeast co-culture at 160 h, Yx/s was 0.56 g biomass/g substrate with whole cell co-culture at the end of 96 h and Yx/s was 0.11 g biomass/g substrate with immobilized yeast co-culture at the end of 120 h.

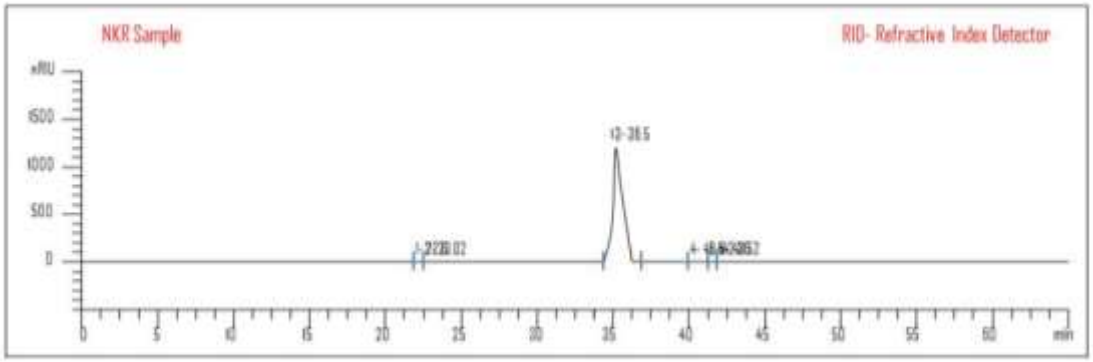

Fig. 4. HPLC chromatogram showing a minute fraction of ethanol of around 0.09 g/L at a retention time of 36.5 min. This was obtained while whole yeast cells were used to co-culture along with *T. harzanium* ATCC® 20846™

The specific growth rate was 0.03 g/h without yeast co-culture at 160 h, specific growth rate was 0.07 g/h substrate with whole cell co-culture at the end of 96 h and it was 0.09 g/h with immobilized yeast co-culture at the end of 120 h. The maintenance coefficients were: 0.04 g/g.h substrate without yeast co-culture at 160 h, 0.02 g/g.h substrate with whole cell co-culture at the end of 96 h and 0.004 g/g.h substrate with immobilized yeast co-culture at the end of 120 h. The process of yeast co-culture to utilize the accumulated glucose in the cellulase production broth has synonymously been termed Simultaneous Saccharification and Fermentation [16].

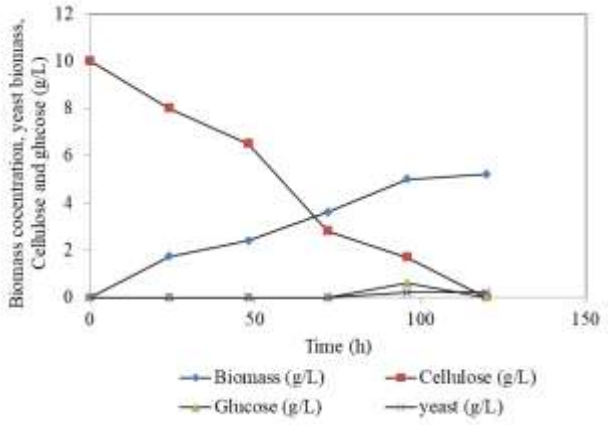

Fig. 5. Cellulase production using immobilized yeast co-culture with *T. harzanium* ATCC® 20846™

Fig. 6(a) shows the production of FPases, 1.946 FPU/mL at 160 h without yeast co-culture, 2.035 FPU/mL at 96 h with yeast whole cells and 1.75 FPU/mL using immobilized yeast cells at 120 h were obtained. Fig. 6(b) shows the production of CMCases, 12.36 CMCase/mL at 160 h without yeast co-culture, 12.72 CMCase/mL at 96 h with yeast whole cells and 11.06 CMCase/mL using immobilized yeast cells at 120 h were obtained. Fig. 6(c) shows the production of Xylanses, 782.7 IU/mL at 160 h without yeast co-culture, 820.5 IU/mL at 96 h with yeast whole cells and 704 IU/mL using immobilized yeast cells at 120 h were obtained. Fig. 6(d) shows the production of Beta-glucosidases, 3126.3 IU/mL at 160 h without yeast co-culture, 3274.8 IU/mL at 96 h with yeast whole cells and 2816.3 IU/mL using immobilized yeast cells at 120 h were obtained.

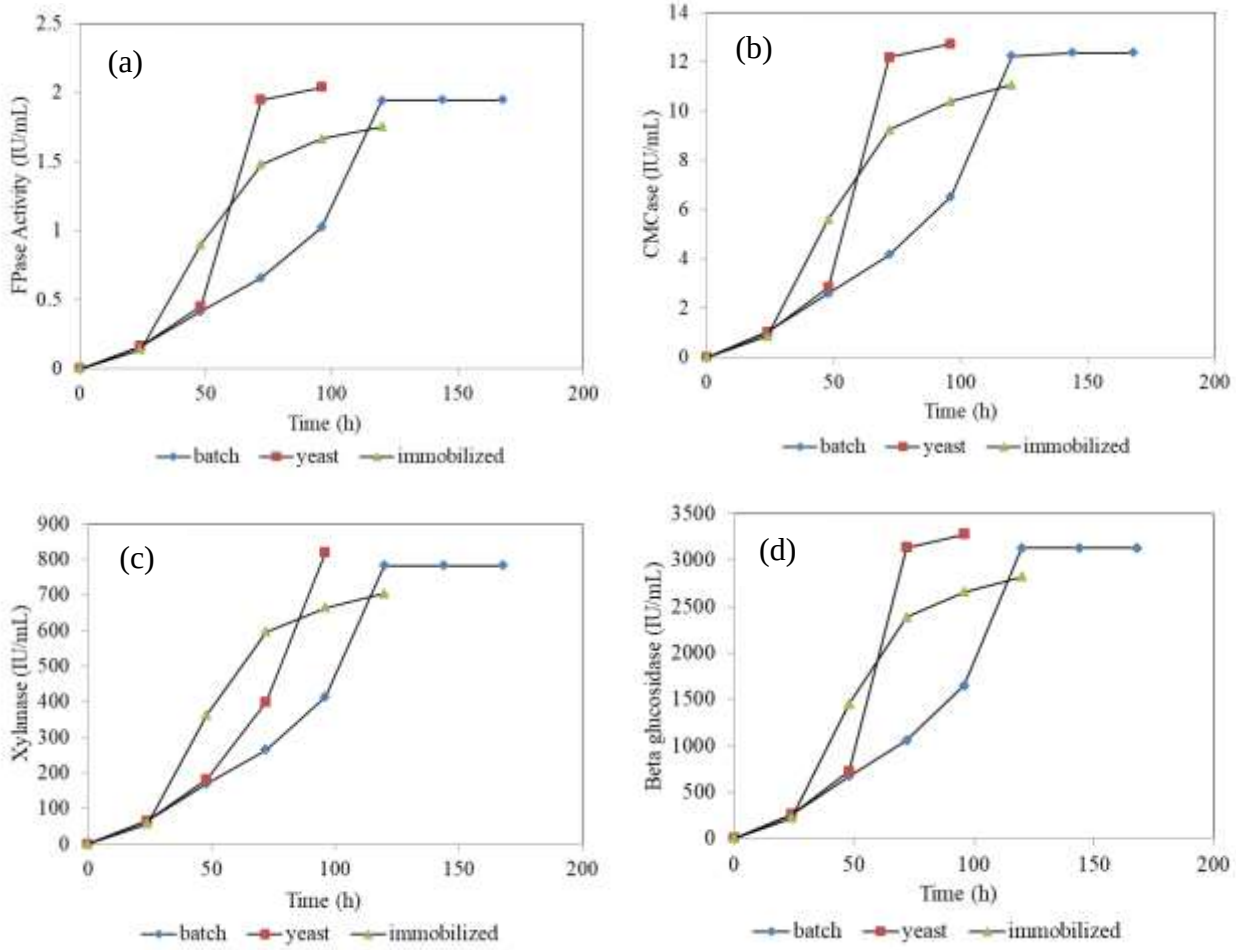

Fig. 6. Enzyme activity using *T. harzanium* ATCC® 20846™ without co-culture, yeast co-culture with *T. harzanium* ATCC® 20846™ and immobilized yeast co-culture with *T. harzanium* ATCC® 20846™ (a) FPASe activity (b) CMCase activity (c) Xylanase activity (d) Beta-glucosidase activity

Some authors refer to this process as Consolidated Bioprocessing where their objective lies in the usage of a single process to convert the lignocellulosic biomass to ethanol. However, in our work we restrict ourselves to calling this method a co-culture based on the fact that our idea has been to increase cellulase production by using a yeast co-culture where the yeast consumes the produced sugars and does not produce ethanol [17].

Though ethanol produced in miniscule quantities, is just an unintended product in this work. Very little quantities of sugars and aerobic conditions within the reactor would divert the yeast metabolism to more biomass formation than ethanol production in contrary to higher concentrations of sugars and aneaerobic conditions [5]. As shown in the Fig. 4 HPLC chromatogram, the glucose is completely depleted from the media which could be because the glucose produced could have been simultaneously consumed by *S. cereviseae* and *T. harzanium* ATCC® 20846™ [18] the production of yeast biomass was minimal as the media supplied very little quantities of glucose while another portion of it was utilized by *T.harzanium* ATCC® 20846™. Another reason

for the slower growth was the lack of certain minimal media components essential for yeast growth [18]. It has also been observed that the rate of cellulose hydrolysis was higher than the rate of glucose consumption and utilization by the yeast [13]. Such a condition would not effectively reduce the glucose accumulation issue. Though cellulases were produced in significant quantities saccharification did not occur to a great extent as most processes of saccharification occur at temperatures of around 50-60°C [16, 18] the temperature for the whole submerged fermentation co-culture was maintained between 28-30°C failing which yeast would'nt survive and grow [17]. A second major advantage was offered by this yeast co-cultre with a cellulolytic fungus. *S. cereviseae* requires a mesophilic condition for a better performance and *T. harzanium* ATCC® 20846™ needs sufficient oxygen to produce cellulases. It was observed that the yeast consumes the reducing sugar providing a suitable environment for *T. harzanium*'s cellulase production while fully mature mycelia of *T. harzanium* would consume the oxygen in the liquid thus creating a suitable microaerophilic/anaerobic environment for the yeast's growth and fermentation [19, 24-26]. This also proves that a symbiotic relationship may exist between the two organisms as they mutually benefit each other during their individual growths.

Growth in Submerged Fermentation

The starter culture in the Vogel's medium contained a significant amount of cellulases. This was used as the starter culture because the cellulases in it may act as an inducer [2] to initiate biomass formation for the culture in the bioreactor's increased volume environment. As expected, we observed quick adaptation and the mycelia started increasing in proportions. Since the agitation was cascaded, it was observed that the agitation increased only to matchup the DO% set point. Agitation with a low shear rate did not cause considerable breakage of the growing hyphae [11]. The aeration was varied manually between 0.5vvm and 1 vvm to keep a check on foaming. Mild concentrations of Poly Propylene Glycol (5-10 ppm) antifoam were used. Addition of excessive antifoam decreases foam and hence influence negatively the K_La, the mass transfer coefficient. It is preferable to operate the reactor without antifoam. However, considering the unmonitored long time operation, a mild concentration of antifoam sufficient to kill the bubbles on the top of the foam was used [14]. The media's viscosity increased after 48 h indicating the secretion of enzymes. The agitator did not cause maximum shear to the hyphae. The hyphal tips produce cellulases [21] and when these hyphae are broken down due to high shear rates, one cannot expect the production of more cellulases. Lengthy hyphae were produced and accumulated in the bottom and on the baffles. A partial solid state growth characteristics were observed when the hyphae accumulated on the baffles and consumed media by secreting more cellulases. The deposited hyphal mass increased in proportion with time. A serious issue of catabolite repression was observed in the fermentation [10-12]. During the DNSA reducing sugar estimation as the values of background sugar in the control sample having just the media along with cellulases was investigated, it was observed that after 72 h, cellobioses and glucose were produced considerably. Cellobioses themselves inhibit and cause catabolite repression during cellulase production [10]. It could be attributed to this reason that there happens to be constancy in the cellulase production after a point of time in the submerged fermentation. This catabolite repression also results in no further usage of cellulose in the media resulting in 2 g/L of cellulose being left as the residual cellulose in the fermentation media after 120 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 presence of a sugar such as glucose will inhibit the further synthesis of cellulases. Hence glucose and cellobioses are considered as negatively impacting substances during the production of cellulases. To utilize maximum amount of cellulose and produce cellulases with enhanced enzyme activities, the stress levels of the fungus needs to be kept high along with the removal of the produced cellobioses and glucoses to provide uninterrupted stress conditions to stimulate higher cellulase yields [4, 20].

Conclusions

T. harzanium ATCC® 20846™ when used for cellulase production suffers catabolite repression due to the accumulation of glucose in the fermentation broth. To counteract this issue whole yeast cells and calcium alginate

immobilized yeast cells were co-cultured with *T.harzanium* ATCC® 20846™ to increase cellulase production. Cellulase activities were increased when yeast co-culture was done. A minute fraction of ethanol was produced as the whole yeast cells fermented a small portion of the sugar into ethanol. The method proves itself as an efficient way to produce cellulases with enhanced activities.

Contribution of Authors

The first and the second authors have equally contributed towards the research work in the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

References

[1] Kuhad RC, Gupta R, Singh A. Microbial Cellulases and Their Industrial Applications. Enzyme Research. 2011;2011:280696.

[2] Arja MO. *Trichoderma resei* for the Production of Cellulases for the Textile Industry. VTT Publications 550. Finland; 2004.

[3] Antoniêto AC, de Paula RG, Castro Ldos S, Silva-Rocha R, Persinoti GF, Silva RN. *Trichoderma reesei* CRE1-mediated Carbon Catabolite Repression in Response to Sophorose through RNA Sequencing Analysis. Curr Genomics. 2016;17:119-31.

[4] Nogueira KMV, Costa MN, de Paula RG, Natividade FCM, Azevedo RR, Silva RN. Evidence of cAMP involvement in cellobiohydrolase expression and secretion by Trichoderma reesei in presence of the inducer sophorose. BMC Microbiology. 2015;15:195

[5] Érika DV, Maria da GSA, Silvio RA. Yeast biomass production: a new approach in glucose-limited feeding strategy. Brazilian Journal of Microbiology. 2013;44(2):551-8.

[6] Lisa G, Paul F, Karen MP. Co-Culture Systems and Technologies: Taking Synthetic Biology to the Next Level. Journal of the Royal Society Interface. 2014;11:20140065.

[7] Bader J, Mast-Gerlach E, Popović MK. Bajpai R, Stahl U. Relevance of microbial coculture fermentations in biotechnology. Journal of Applied Microbiology. 2009;109(2):371-87.

[8] Ghose TK. Measurement of Cellulase Activities. Pure App Chem. 1987;59(2), 257-68.

[9] Ahamed A, Vermette P. Culture-based strategies to enhance cellulase enzyme production from *Trichoderma reesei* RUT-C30 in bioreactor culture conditions. Biochemical Engineering. 2008;40:399-407.

[10] Yi-Heng PZ, Lee RL. Regulation of Cellulase Synthesis in Batch and Continuous Culture of *Clostridium thermocellum*. Journal of Bacteriology. 2005;187(5):99-106.

[11] Bakri Y, Akeed Y, ThonartP. Comparison between Continuous and Batch Processing to Produce Xylanase by *Penicillium canescens* 10-10c. Brazilian Journal of Chemical Engineering. 2012;29(3):441-448.

[12] Navnit kumar R, Sambavi TR, Jason Charles S, Renganathan S. A Novel Spectroscopic method for Fungal Growth Estimation. International Journal of Industrial Engineering. 2017;1(9):282-89.

[13] Kádár Z, Szengyel Z, Réczey K. Simultaneous saccharification and fermentation (SSF) of industrial wastes for the production of ethanol. International Crops and Products. 2004;20(1):103-10

[14] Seletzki MJ. Thesis. Process Development and Scale-up from Shake Flask to Fermenter of Suspended and Immobilized Aerobic Microorganisms. RWTH Aachen University. 2007.

[15] Liu ZH, Qin L, Zhu JQ, Li BZ, Yuan YJ. Simultaneous Saccharification and Fermentation of Steam Exploaded Corn Stover at High Glucan Loading and High Temperature. Biotechnology for Biofuels. 2014;7:167.

[16] Hari Krishna S, Chowdary GV. Optimization of Simultaneous Saccharification and Fermentation for the Production of Ethanol from Lignocellulosic Biomass. Journal of Agriculture and Food Technology. 2000;48(5):1971-76.

[17] Swain MR, Mishra J, Thatoi H. Bioethanol Production from Sweet Potato (*Ipomoea batatas* L.) Flour using Co-Culture of *Trichoderma* sp. and *Saccharomyces*

cerevisiae in Solid-State Fermentation. Brazilian Archives of Biology and Technology. 2013;56(2):171-9.

[18] Park EY, Naruse K, Kato T. One–pot Bioethanol Production from Cellulose by co-culture of *Acremonium cellulolyticus* and *Saccharomyces cereviseae*. Biotechnology for Biofuels. 2012;5:64.

[19] Zuroff T, Salvador Barri S, Curtis WR. Consortia Mediated Bioprocessing of Cellulose to Ethanol with a Symbiotic *Clostridium phytofermentas*/yeast CO-culture. Biotechnology for Biofuels. 2013;6(59).

[20] Schuster A, Tisch D, Seidl-Seiboth V, Kubicek CP, Schmoll M. Roles of Protein Kinase A and Adenylate Cyclase in Light-Modulated Cellulase Regulation in Trichoderma resei. Applied and Environmental Microbiology. 2012;78(7):2168-78.

[21] Willia Schneider WDH, Reis LD, Camassola M, Dillon AJP. Morphogenesis and Production of Enzymes by Penicillium echinulatum in Response to Different Carbon Source. BioMed Research International. 2014;2014:254863.

[22] Tomé MF. Direct Cost of Reprocessing Cotton-woven Surgical Drapes: a Case Study. Rev Esc Enferm USP. 2015;49(3):488-94.

[23] Nehmea N, Mathieub F, Taillandier P. Impact of the co-culture of Saccharomyces cerevisiae–Oenococcus oeni on malolactic fermentation and partial characterization of a yeast-derived inhibitory peptidic fraction. Food Microbiology. 2009;27(1):150-57.

[24] Shahzadi T, Anwar Z, Iqbal Z, Anjum A, Aqil T, Bakhtawar, Afzal A, Kamran M, Mehmood S, Irshad M. Induced Production of Exoglucanase, and β-Glucosidase from Fungal Co-Culture of *T. viride* and *G. lucidum*. Advances in Bioscience and Biotechnology. 2014;5(5):426-33.

[25] Hanly TJ, Henson MA. Dynamic metabolic modeling of a microaerobic yeast co-culture: predicting and optimizing ethanol production from glucose/xylose mixtures. Biotechnology for Biofuels. 2013;6:44.

[26] Paranthaman R, Vidyalakshmi R, Murugesh S, Singaravadivel K. Effects of Fungal Co-Culture for the Biosynthesis of Tannase and Gallic Acid from Grape Wastes under Solid State Fermentation. Global Journal of Biotechnology and Biochemistry. 2009;4:29-36.

****

