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ORIGINAL ARTICLE

Ethanol production by a filamentous fungal strain *Byssochlamys fulva AM130* under alternating aerobic and oxygen-limited conditions

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Abstract

Byssochlamys fulva AM130, a novel strain of filamentous fungus, could produce ethanol from glucose, xylose, and alkali pretreated rice straw (PRS), while the efficiencies were very low with PRS. Ethanol production of 11.84 g/L was attained by the fungus when grown in glucose, indicating that the limitations while growing on PRS were related to low hydrolytic efficiency. Enzyme profiling of the fungus showed 365 IU/ml of beta-glucosidase and 89 IU/ml of xylanase activity, while endoglucanase and filter paper activity were negligible, which accounts for the low hydrolytic efficiency. The fungus could survive for extended periods under oxygen-limited conditions and produce ethanol. The fungal mycelia could also be used for repeated cycles of anaerobic fermentation, wherein the ethanol yield improved with each consecutive cycle.

Keywords *Byssochlamys fulva* · Ethanol · Consolidated bioprocessing · Cellulase · Pentose fermentation · Beta-glucosidase

Introduction

One of the major challenges in second-generation ethanol production is the lack of organisms that can efficiently ferment both C6 and C5 sugars. This often requires the use of engineered microbes that can ferment both sugars, co-fermentation using C6 and C5 fermenting organisms or modifying the process itself to generate separate C6 and C5 streams whose value addition can be independent of each other [11, 24]. Most of the current common pretreatment strategies, like dilute alkali, alkaline peroxide, ammonia fiber explosion (AFEX) etc., leaves the hemicellulose intact in the solid fraction, while the lignin is removed. During the hydrolysis step, the enzymes act on both the hemicellulose and cellulose liberating a mixture of both C6 and C5 sugars. Typical yeasts used in commercial fermentation, the strains of *Saccharomyces cerevisiae,* are not capable of C5 fermentation with the results that, after the fermentation, the available C5 sugars are unutilized and often goes into distillation. Natural C5 fermenting organisms, like *Pichia stipitis,* are inhibited by acetic acid and lignin derivatives and their ethanol tolerance is less compared to *S. cerevisiae* [8].

It is known that certain fungi, especially those belonging to the genera *Aspergillus, Paecillomyes, Mucor, Rhizopus, Fusarium,* and *Trichoderma,* can directly ferment cellulose to ethanol, albeit with low efficiency [2]. The ethanol productivities of these fungi, though considered low by standards of ethanol fermenting yeasts, are high for organisms considered as non-fermentative [22]. Fungi are comparatively tolerant to fermentation inhibitors, like acetic acid, and lignin breakdown products and they are capable of fermenting C5 sugars, in addition to glucose [15]. Most of these fungi are aerobic, but exposure to anaerobic conditions results in inhibition of glycolysis and consequently a switch over to fermentation mode [5]. The ability to sustain growth and survive under anaerobic conditions, and the tolerance of

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these fungi to ethanol which they produce under anaerobic conditions have been relatively understudied and so is their potential to directly produce ethanol from lignocellulosic biomass. Recent studies have indicated the potential of using edible filamentous fungi for valorization of spent liquors and C5-rich streams from grain and lignocellulosic biorefineries where edible fungal protein is generated along with ethanol fermentation [17, 18]. Apparently, most fungi are capable of utilizing cellulose and hemicellulose-derived sugars, but their ability to synthesize lignocellulolytic enzymes varies. While all fungi may not be ideal for a consolidated bioprocessing (CBP) scenario, ability to utilize C6 and C5 sugars for ethanol production, tolerance to ethanol, longer survival in anaerobic mode, and tolerance to potential inhibitors in biomass hydrolyzates makes a select few of them potential candidates for integration into second-generation biofuel production scenario. They could be used post yeast fermentation of hydrolyzates to convert the unutilized C5 sugars and in the process generating a protein-rich residue with further conversion value.

Previous studies had indicated the potential of a filamentous fungal isolate *Byssochlamys fulva* AM130 which can produce ethanol under anaerobic conditions [13]. *Byssochlamys* are soil fungi frequently associated with spoilage of food especially canned fruits, and are known to produce pectinases. They produce heat-resistant ascospores and are known to grow under low oxygen tension and sometimes considered a health risk due to production of mycotoxins [9]. The present study was conducted to evaluate the strain in terms of its ability to use C6 and C5 sugars, ethanol tolerance, survival under anaerobic mode, and the production of ethanol.

Materials and methods

Organism and preparation of inocula

Byssochlamys fulva AM 130 available in the culture collection of CSIR-National Institute for Interdisciplinary Science and Technology was used for this study. The culture was maintained on Potato Dextrose Agar (PDA) slants and was periodically sub-cultured. Spore inoculum at a concentration of 1×10^8 spores/ml (in 0.005 g/L Tween 80 solution) was used for preparing the mycelial inoculum /biomass used in this study. One milliliter of the spore suspension was inoculated into 500 ml Erlenmeyer flasks containing 100 ml of Mandels and Weber (M&W) medium [12] containing 50 g/L glucose as carbon source. Flasks were incubated on a shaker incubator at 30 ± 2 °C and 200 rpm agitation for 96 h. Mycelial pellets were harvested by filtration through sterile nylon sieves and were allowed to drain all free water. The wet drained mycelial biomass volume was measured using a sterile graduated vessel and was used at 10% v/v (5 cc /50 ml) as inoculum in the fermentation studies.

Fungal cultivation in different carbon sources and alcohol production

Mandels and Weber medium [12] with the following composition in g/L—KH_2PO_4, 2; Urea, 1.3; $CaCl_2.2H_2O$, 0.3; $MgSO_4.7H_2O$, 0.3; $(NH_4)_2SO_4$, 1.4; Peptone, 0.75; Yeast Extract, 0.25; $FeSO_4.7H_2O$, 0.02; $MnSO_4.7H_2O$, 0.006; $ZnSO_4.7H_2O$, 0.014; and $CoCl_2.2H_2O$, 0.008—was used for culturing of fungi. Three different carbon sources—glucose, xylose, or alkali pretreated rice straw (PRS)—was evaluated for fungal cultivation and ethanol production. Alkaline pretreated rice straw was prepared as previously described [14]. Carbon source was added as per the experimental design at the required concentration into the basal medium for each study. pH of the medium was adjusted to 5.0 using 1 N HCl or 1 N NaOH. Erlenmeyer Flasks (250 ml) containing 50 ml M&W medium with appropriate carbon source were inoculated with 5.0 cc volume of mycelial inoculum prepared as above and were incubated under stationary conditions at room temperature (30 ± 2 °C) to form the oxygen limited/anaerobic phase. After 48 h, 50 ml of fresh medium containing the same carbon source as the original at 50 g/L concentration was added and the aerobic mode was initiated by agitation at 200 rpm. After a 48 h aerobic cultivation, another 50 ml of medium as above was added and the culture was maintained stationary to achieve oxygen limited/anaerobic conditions. Samples were taken at regular intervals and the total sugar and ethanol concentrations were determined.

Evaluation of the fungal tolerance to oxygen-limited condition and growth during oxygen-limited phase

Five sets of 250 ml flasks each containing 50 ml of M&W medium with 50 g/L glucose were inoculated with 5.0 cc of mycelial inoculum prepared as above, and were incubated as stationary cultures at 30 ± 2 °C for 48, 72, or 96 h to assess fungal tolerance to oxygen-limited/anaerobic condition. Experiments were conducted with a constant aerobic phase duration of 48 h, and the duration of each oxygen-limited phase was varied as mentioned above. At the start of each phase switching, additional 50 ml of fresh medium was added, so that carbon source was not limiting. In all the cases, a total of three cycles of oxygen-limited fermentation were performed. Samples were taken at regular interval and were analyzed for sugars and ethanol. Growth was estimated by monitoring biomass wet weight. For this, the experiments were carried out using 50 g/L glucose as carbon source and using a 96 h oxygen-limited phase and 48 h aerobic phase. Biomass was collected at the end of each phase through sterile filtration on a nylon sieve and after a brief wash in sterile

medium, which was weighed aseptically in an analytical balance. The biomass was returned to the flask and an additional 50 ml medium was added with glucose concentration adjusted to obtain a final concentration of 50 g/L glucose in the medium. Experiments were performed with alternating 96 h oxygen-limited phase and 48 h aerobic phase. Samples were withdrawn at regular intervals and the sugar and ethanol concentrations were determined.

Ethanol tolerance of fungus

For the ethanol tolerance test, 5 cc of wet mycelia of *B. fulva* was added to 50 ml M&W medium in 250 ml Erlenmeyer flasks and was supplemented with 4, 8, 12, 16, 20, 24, 28, 32, 36, or 39 g/L ethanol. The cultures were incubated aerobically at 25 ± 2 °C with150 rpm agitation for 72 h. After the incubation, the mycelia were separated by filtration and were re-suspended in 50 ml of fresh medium without ethanol. Morphological and microscopic observations were carried out to check for damages.

Enzyme profiling of the fungus

Wet mycelia of *B. fulva* prepared as above was inoculated at 10% v/v level in 50 ml of M&W medium containing 25 g/L PRS as carbon source, and was incubated for 72 h at 30 °C and 200 rpm agitation. At the end of incubation, samples were collected and assays for cellulase, Carboxymethyl cellulase /endoglucanase (CMCase) and beta-glucosidase (BGL) were conducted as per the IUPAC method [6]. Xylanase activity was assayed as per the method of Bailey et al. [4].

Effect of enzyme supplementation on ethanol production by *B. fulva* on PRS

Fully grown mycelial inoculum (5 cc volume) prepared as mentioned above was used to inoculate 100 ml of M&W medium containing 50 g/L PRS in 500 ml Erlenmeyer flasks. Commercial Cellulase enzyme (Biopol, Zytex Inc, India) was added at 10 FPU/g concentration flasks after filter sterilization. The flasks were capped and were incubated at room temperature (30 ± 2 °C) with mild agitation to allow mixing. Fermentation broth was collected at 0, 12, 24, 36, 48, 72, and 96 h of the oxygen-limited phase. Samples were centrifuged at 10,000 rpm for 10 min at 4 °C and the supernatant was used for testing biomass hydrolysis as sugar release and the ethanol production.

Estimation of sugars and ethanol

Estimation of total sugars was performed by the DNS method [16]. Glucose and xylose were estimated using HPLC (Shimadzu Prominence UFLC) using a Phenomenex Resex ® column (Phenomenex, India) and a Refractive Index detector following the NREL method [23]. The analysis was performed at a column temperature of 55 °C and detector temperature of 40 °C. Mobile phase used was 0.005 M H_2SO_4 with a flow rate of 0.6 ml/min. Ethanol in the samples was analyzed by a gas chromatograph (Shimadzu GC 2014). Poropak Q ® column was used for separation by maintaining the oven temperature as a gradient with rise in temperature from 50 to 200 °C at the rate of 8 °C/min and were detected by flame ionization detector (FID). Injector and detector temperature were 150 °C and 250 °C, respectively. Injection volume was 2.0 µl.

Results and discussion

Sugar utilization and ethanol production by *Byssochlamys fulva* AM130

Utilization of glucose and xylose by *Byssochlamys fulva* was studied under alternating aerobic and oxygen-limited conditions. Under aerobic cultivation with 5% of either glucose or xylose as carbon source, the consumption of glucose (51%) was higher compared to xylose (36%) (Fig. 1). During the oxygen-limited phase which lasted 48 h, sugar consumption by the fungus was lower than that in aerobic phase. Ethanol production at the end of oxygen-limited phase was 2.82 g/L (0.36% v/v) in media containing glucose and 1.59 g/L (0.19%v/v) in xylose (Data not shown). The measured consumption of glucose was low for the oxygen-limited phase, to account for the ethanol yield obtained, which indicated that there could be ethanol production during the aerobic phase also.

Evaluation of ethanol at the end of aerobic phase for glucose grown culture indicated that there is 2.1 g/L (0.263% v/v) ethanol in the medium. Ethanol production normally happens in the absence of oxygen, when the pyruvate produced during glycolysis is diverted to anaerobic fermentation to produce ethanol and carbon dioxide. However, it is also known that at least in yeast, anaerobic fermentation which results in formation of ethanol is the preferred mode of respiration even in the presence of oxygen [10]. Yeasts, therefore, are capable of alcohol production even in the presence of considerable amounts of oxygen. It could be speculated that during fungal growth in shake flasks, microaerobic conditions may be generated inside the mycelial pellets. Experiments were therefore conducted with 96 h aerobically grown mycelial inoculum for alternating oxygen-limited and aerobic phases to monitor consumption of sugars and ethanol production in both phases.

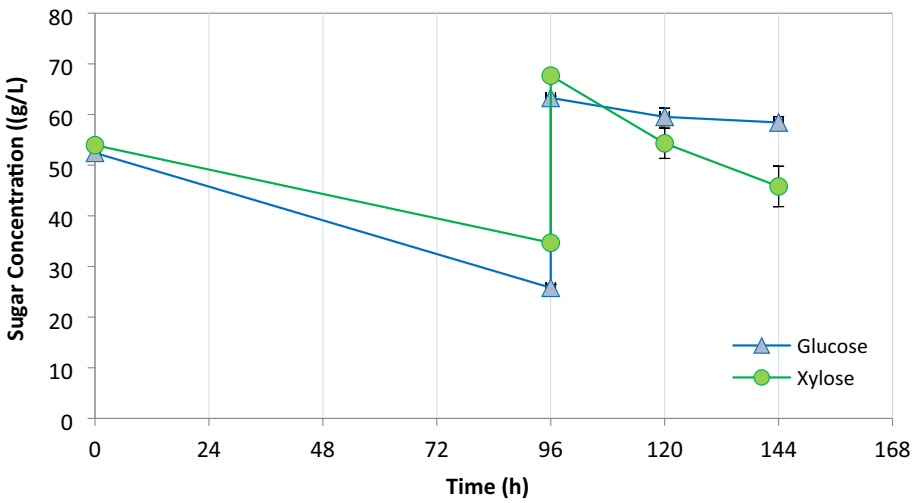

Fig. 1 Utilization of sugars by *Byssochlamys fulva*. Legends: 0–96 h: aerobic phase, 96–144 h: oxygen limited

Ethanol production by *B. fulva* from glucose or xylose under alternating oxygen-limited and aerobic conditions

It was observed during the initial trials that there was a difference in the consumption of glucose and xylose between aerobic and oxygen-limited phases. Studies were therefore conducted to evaluate the preference in consumption of sugars by the fungus and ethanol production when grown on these sugars.

Data shown in Fig. 2a indicated that the consumption of glucose was uniform, regardless of the mode of cultivation (aerobic or oxygen limited), and at the end of the second oxygen-limited phase, the yield of ethanol was 3.74 g/L (0.474% v/v). About 82% of the glucose was consumed by the organism, together in the oxygen-limited and aerobic phases. Compared to glucose, the consumption of xylose was slightly lesser at 77% (Fig. 2b). However, the ethanol yield at the end of the second oxygen-limited phase was only 1.03 g/L (0.131% v/v). This indicated that glucose is indeed the preferred carbon source, while xylose can also be used by the organism. In either case, production of ethanol during aerobic phase was negligible.

Ability of the fungus to utilize both glucose and xylose as carbon sources under both aerobic and oxygen-limited conditions and to produce ethanol during the oxygen-limited phase indicated a possibility for integration in lignocellulose conversion. To further study the suitability of the fungus in such a process scenario through production of sugars from biomass by elaboration of biomass-hydrolyzing enzymes, studies were conducted on sugar release and ethanol production. In this case, sugar consumption was difficult to be monitored as the sugars produced through enzymatic action may be consumed by the fungus itself as soon as it is formed. Nevertheless, glucose generation in excess of that what is being consumed was noted as free sugars were detected at every time point sampled (Fig. 2c). Here, the sugar concentration increased slightly from 0.8 g/L at 0 h of the first oxygen-limited phase and reached 1.23 g/L at 48 h of incubation. Ethanol concentration also increased during this period to 0.434 g/L (0.06% v/v). During the subsequent aerobic phase, the sugar concentration increased after an initial drop to 0.8 g/L (due to addition of fresh medium at the start of aerobic phase) to 1.05 g/L. However, the ethanol concentration dropped possibly because of the dilution and no new production.

The sugar concentration increased during the second oxygen-limited phase up to 120 h when it reached the peak value of 2.02 g/L, after which it dropped to 1.88 g/L in the next 24 h. This also coincided with fresh ethanol production, which was indicated by increase in ethanol concentration from 0.02 to 0.10 g/L. While the ethanol production was very low, this may be attributed to the low amount of sugars available, which in turn could be due to the low levels of biomass-hydrolyzing enzymes. It may be noted that that when sugar was available, the ethanol production showed an increase and the upward trend continued till 48 h—the maximum tried. Increased ethanol production with increase in duration of the oxygen-limited phase indicated that optimal duration of this phase could be higher, and therefore, experiments were conducted for optimizing the duration of oxygen-limited phase.

Tolerance of *B. fulva* to anaerobic oxygen-limited cultivation and growth during oxygen-limited phase.

The ability to survive and possibly grow anaerobically is the key to the use of filamentous fungi in ethanol production. In the case of *Trichoderma reesei*, there is repression of enzymes involved in glycolysis when grown anaerobically [5], In *Fusarium oxysporum*, it has been demonstrated that

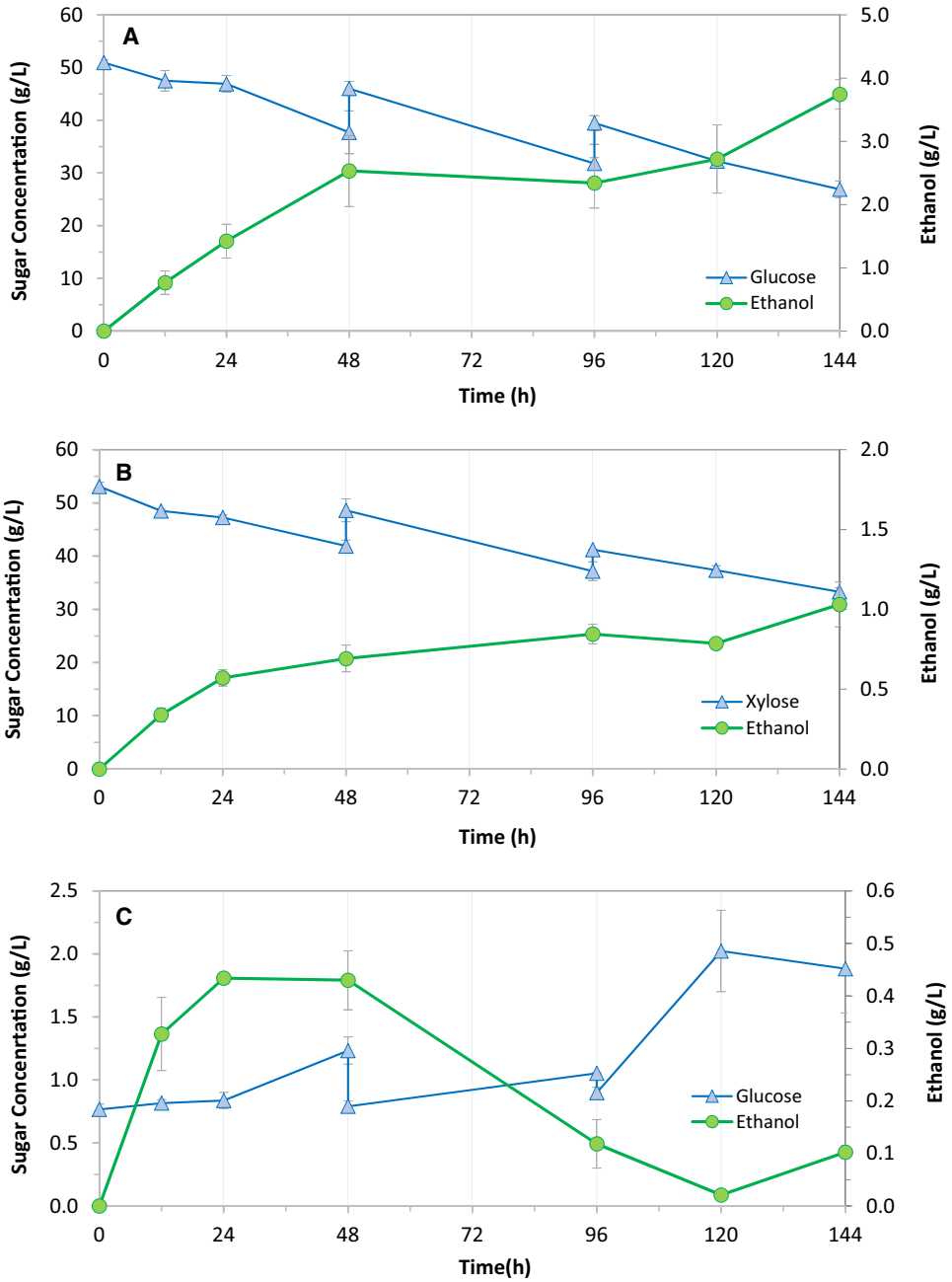

Fig. 2 Ethanol production by *Byssochlamys fulva* utilizing different carbon sources. Legends: (**a**) glucose as carbon source (**b**) Xylose as carbon source (**c**) alkali pre-treated rice straw as carbon source. | 0–48 h and 96–144 h—oxygen limited phase, 48–96—aerobic phase

there is a blockage of the TCA cycle when the fungus was grown anaerobically [19]. Such limitations have resulted in the studies on fungal alcohol production to be planned in two phases—an aerobic growth phase where the fungus is allowed to grow and build up enough biomass and produce enzymes required for the hydrolysis of the substrates, and an anaerobic fermentation phase where the aerobically grown mycelia are used for ethanol production by switching to anaerobic mode. This allows for enzymatic digestion of biomass and the use of the sugars for ethanol production [22]. Provided there are enough sugars, the efficiency of ethanol production is determined by how long the fungus can survive under anaerobic mode, since a longer duration might allow better adaptation to the anaerobic conditions and increased conversion of the sugars to ethanol. The ideal duration of anaerobic/oxygen-limited phase for *B. fulva* was determined by extension of the oxygen-limited phase from 48 to 96 h in 24 h increments as separate experiments. At the start of each phase, 50 ml of fresh medium with 50 g/L glucose was added to the culture. Results shown in Fig. 3a, b, C indicated that increased duration of oxygen-limited phase indeed resulted in enhanced ethanol production and the fungus could survive oxygen-limited conditions for at least 96 h until switched to the aerobic phase.

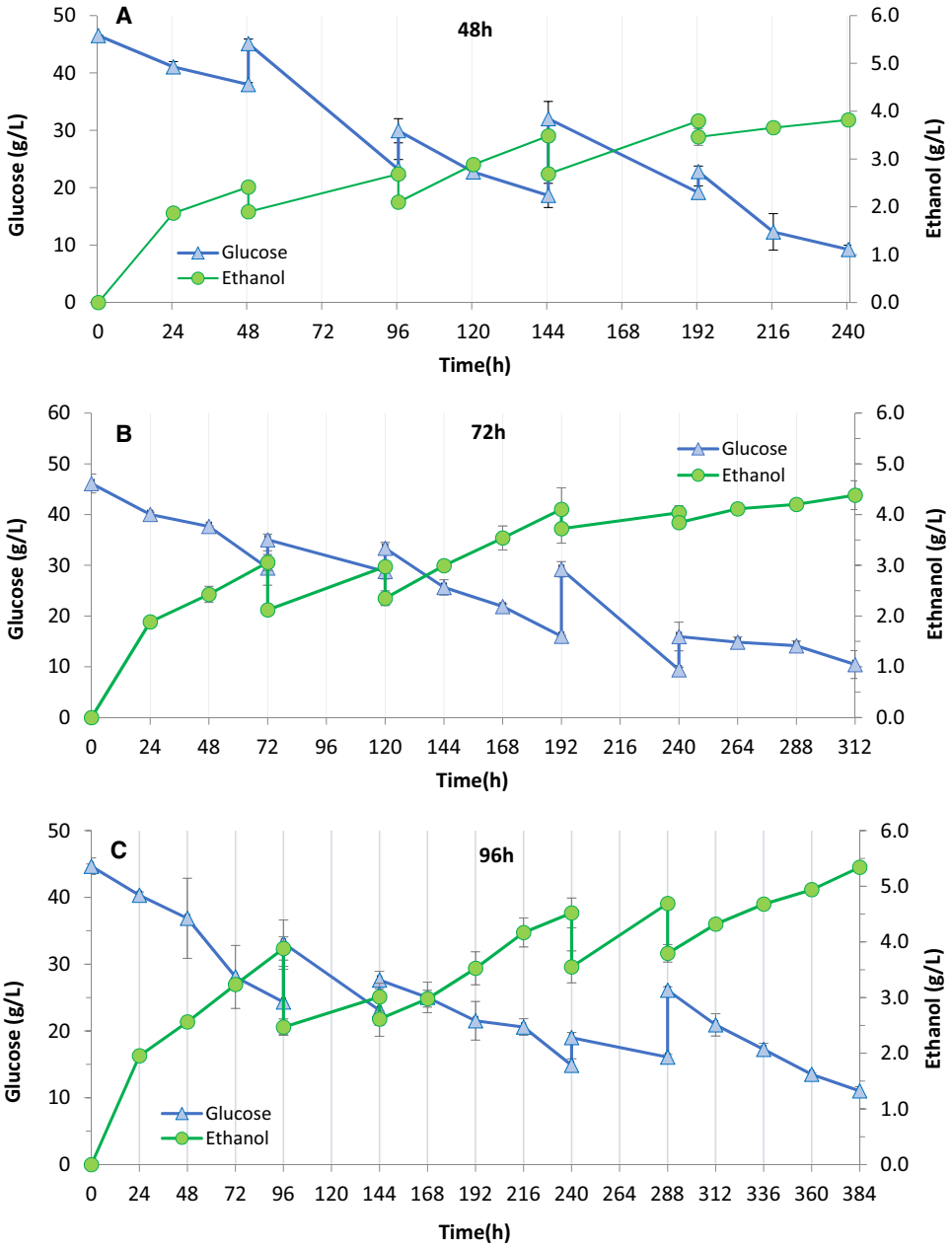

Fig. 3 Survival and ethanol production *Byssochlamys fulva* under varying duration of oxygen limitation. Legends: (**a**) 48 h oxygen limited phase (**b**) 72 h oxygen limited phase (**c**) 96 h oxygen limited phase | **a**: oxygen limited phase—0–48 h, 96–144 h and 192–242 h, aerobic phase—48–96 h and 144–192 h, **b**: oxygen limited phase—0–72 h, 120–192 h and 240–312 h, aerobic phase—72–120 h and 192–240 h, **c**: oxygen limited phase—0–96 h, 144–240 h and 288–384 h, aerobic phase 96–144 h and 240–288 h

While the maximal ethanol yield at the end of three oxygen-limited cycles was 3.79 g/L (0.48%v/v) for the experiments with 48 h oxygen-limited phase, the ethanol yields after three oxygen-limited cycles for 72 h and 96 h were 4.42 g/L (0.56% v/v) and 5.34 g/L (0.677% v/v), respectively. Better performance in the case of increased duration of oxygen-limited phase which improved with each cycle indicated not only the survival of the fungal mycelium but also their potential to grow in anaerobic mode. It was also noted that the ethanol production was less efficient compared to the consumption of sugars, which indicated the use of the sugars for growth. Ability of the fungus to tolerate ethanol also determines its maximal ethanol production capacity. The fungus was therefore evaluated for its ability to grow under oxygen-limited conditions and the tolerance toward long-term exposure to ethanol.

Evaluation of the growth of *B. fulva* during oxygen-limited phase.

Fungal growth was monitored for a total of 384 h which included three 96 h oxygen-limited phases and two 48 h aerobic phases in M&W medium with glucose as carbon source. At the start of each phase, glucose concentration was adjusted to 50 g/L, so that sugar does not become limiting. Growth was measured as the increase in wet weight

of mycelial pellets at the end of each phase. Sugar and ethanol concentrations were measured at 24 h intervals. On an average about 42% of the sugar was consumed in each cycle. Glucose consumption was 39% for the aerobic phase, whereas it was 44% for oxygen-limited phase. The culture could grow under both aerobic and oxygen-limited conditions, and the average increase in biomass during the aerobic phase was almost double that of oxygen-limited phase (Table 1). Also, with each progressive aerobic phase, the increase in biomass was higher. It is already known, at least in *Fusarium oxysporum,* that the metabolic pathways active during aerobic and anaerobic conditions are different with the TCA cycle being active under aerobic conditions and the EMP pathway under anaerobic conditions. A high efflux of glyceraldehyde 3 phosphate and fructose 6 phosphate from pentose phosphate pathway to the EMP pathway under anaerobic conditions is believed to be responsible for the high ethanol production by the organism [20]. A major limitation of *F. oxysporum,* which is proposed as one of the most efficient ethanol-producing filamentous fungus, is its slow rate of growth [1]. As observed here, *B. fulva* seems to have an advantage of high growth rate, which increased with each consecutive aerobic phase. While ethanol production was very low during the initial cycles, the efficiency of ethanol production increased with each consecutive oxygen-limited phase (Table 1). By end of the third oxygen-limited phase, the conversion efficiency based on total sugar at the start of the phase improved to 26% and the final ethanol concentration achieved was 11.68 g/L. The ethanol yield improved from 0.05 g/g during the first anaerobic cycle to 0.13 g/g in the final oxygen-limited cycle. While it took nearly 16 days to achieve this, there was a constant improvement in ethanol yields with increasing number of cycles. A high biomass concentration has been considered critical for improving the ethanol yields [3]. Ethanol yields ranging from 0.18 to 0.46 g/g have been documented for strains of *Fusarium oxysporum*, one of the best fungal producers of ethanol [21]. Nair et al.[18] reported an ethanol yield of 0.23 g/g for *Neurospora intermedia* in a mixture of dilute acid pretreated and enzymatically hydrolyzed wheat straw and thin stillage from the first-generation ethanol process. A more recent study, targeting direct fermentation of lignocellulosic biomass using a mixed consortium of fungi, has shown ethanol yields of 0.037, 0.40, and 0.41 g/g for the fungi *Bjerkandera adusta, Fomitopsis palustris, and Schizophyllum commune,* respectively, while using glucose as the carbon source [7]. These were the highest yields for the fungi, and the duration taken for achieving these yields were 18, 28, or 6 days, respectively, for *B. adusta, F. palustris* and *S. commune*. Apparently, there is a lot of variability between the fungi, both in their fermentation abilities and the time taken for achieving maximal yields of ethanol. The yield obtained in the current study, though, is at the lower

Table 1 Growth, sugar consumption and ethanol production by *B. fulva* cultivated under alternating anaerobic and anaerobic phases

Phase	Time (h)	Volume at the start and end of phase (ml)	Fungal Biomass (g)	Sugar Conc. (g/L)	Total Sugar (g)	Sugar consumed during the phase (g)	Ethanol Theoretical max (g)	Ethanol Conc. Obtained (g/L)	Total ethanol (g)	Ethanol produced during the phase (g)	Ethanol Yield (g/g) [Conversion efficiency (% of theoretical)]
Oxygen limited 1	0	50	1.26	48.25	2.41	0.98	1.23	0.00	0.00	0.12	0.05 [9.49]
	96	50		28.68	1.43			2.34	0.12		
Aerobic 1	96	100	2.13	50.10	5.01	1.87	2.56	1.28	0.13	0.09	0.02 [3.38]
	144	100		31.37	3.14			2.15	0.21		
Oxygen limited 2	144	150	4.15	52.38	7.86	3.56	4.01	1.64	0.25	0.72	0.09 [17.87]
	240	150		28.65	4.30			6.41	0.96		
Aerobic 2	240	200	6.23	50.31	10.06	4.01	5.13	3.89	0.78	0.47	0.05 [9.23]
	288	200		30.27	6.05			6.26	1.25		
Oxygen limited 3	288	250	9.73	50.15	12.54	5.77	6.39	5.10	1.28	1.64	0.13 [25.70]
	384	250	10.34	27.09	6.77			11.68	2.92		

end of the spectrum; it may be observed that the yield has kept on increasing with each cycle. This indicates the potential for further improvement in sugar consumption, and its conversion to ethanol through the use of acclimatized fungal mycelia as was used here.

Tolerance to ethanol and enzyme production by *B. fulva* AM130

With increase in concentration of ethanol from 0.5 to 5.0% v/v, there was no noticeable difference in growth, and only a slight decrease in the size of the mycelial pellets was noticed. The cells exposed to ethanol continued to grow when supplemented with fresh medium and cultivated under oxygen limitation, indicating survival and metabolic ability (data not shown). There was no noticeable change in mycelial morphology under microscopic observation (Fig. 4). The studies on ethanol tolerance of ethanol-producing fungi are limited. In *Fusarium oxysporum,* it has been reported that the production of mycelia and specific growth rate are affected by the presence of ethanol in the medium. Under limited aeration conditions, ethanol production ability of the fungus was completely inhibited at 45 g/L ethanol [3]. Tolerance to ethanol by *B. fulva* and ability to recover and grow normally could be an advantage, if the fungus is used in a lignocellulosic ethanol production scenario.

Production of biomass-hydrolyzing enzymes is an important property for the use of filamentous fungi to be integrated into a lignocellulose to ethanol process. Apparently, *B. fulva* AM130 may be speculated to have this ability as it could ferment pretreated rice straw (PRS) to ethanol, albeit with lower efficiency. The fungus could produce 0.463 g/L of sugars when incubated aerobically with PRS for 48 h, indicating that it is capable of elaborating biomass-hydrolyzing enzymes. However, the sugar yields were lower, indicating that an optimal enzyme mixture may not have been elaborated by the culture. Enzyme profiling of the culture supernatant of 72 h PRS grown culture showed only beta-glucosidase (BGL) and xylanase activities, which were 365 IU/ml and 89 IUs/ml, respectively. Filter paper activity and CMCase (endoglucanase activity) were negligible. This explains the lower ethanol yields when the culture is

Fig. 4 Tolerance of *Byssochlamys fulva* to ethanol and mycelial growth after exposure. **a** Fungal culture after 72 h incubation in media containing 5% ethanol. **b** Same cultures after transfer to fresh medium and cultivation under aerobic condition for 24 h. **c**, **d**—Microscopic images showing fungal morphology when cultivated in presence of ethanol and without it respectively

cultivated with only PRS as the carbon source. The fungus which could achieve 11.68 g/L (~ 1.5%) of ethanol when supplemented with glucose was limited by the low level of exo- and endo-glucanases and it could be speculated that a higher level of cellulase expression may help in achieving higher ethanol yields. A study was therefore planned to determine the ethanol yield from PRS when supplemented with cellulase.

Hydrolysis and fermentation of PRS by *B. fulva* with cellulase supplementation

Since the absence or near absence of exo- and endo-glucanases was the major limitation for *B. fulva* in the production of ethanol from PRS, studies were conducted with 10 FPUs/g supplementation of a commercial cellulase preparation at the start of the oxygen-limited phase. The oxygen-limited phase was continued for 96 h followed by a 48 h aerobic phase and followed by another oxygen-limited phase. The concentration of total sugars and ethanol in medium was monitored every 24 h. Supplementation of enzyme at the start of fermentative phase indeed resulted in an increase in sugar concentration to 8.4 g/L in 24 h, which also resulted in an ethanol production of 3.63 g/L. In 72 h, the ethanol concentration reached the peak value of 6.47 g/L. The sugar concentration declined starting from 48 h and reached the lowest value in 96 h after which there was no significant change in it (Fig. 5). The same period (48–96 h) also marked the increase in ethanol concentration which corresponded to the decline in sugar concentration. While the peak ethanol concentration was achieved at 72 h, the ethanol concentration at 96 h was almost similar at 6.23 g/L. Ethanol concentration declined drastically during the aerobic phase, possibly because of evaporation loss and the dilution. It did not recover as there was no improvement in sugar yields, indicating loss of enzyme activity, or consumption of sugars for growth. However, the sugar concentration did not drop to zero, either because of the lack of consumption or because it was being matched by enzymatic release of sugars from PRS.

It was speculated that if the reduction in sugar concentration during aerobic phase is due to its consumption by the fungus and not because of the loss of enzyme activity, extending the oxygen-limited phase may increase the ethanol yield. Interestingly, the approach seemed to work as the ethanol production increased from 5.13 g/L at 96 h to 6.23 g/L at 168 h (Fig. 6). The drop in sugar concentration correlated well with the increase in ethanol yield, till about 120 h, after which the sugar concentration seemed to stabilize at ~ 1 g/L, indicating a balance between production and consumption of sugars. Ethanol concentration seemed to increase slightly, indicating that the sugar consumed was also being used for ethanol production. The major limitation of the organism therefore seems to be the low-level expression of exo- and endo-glucanases which could be modulated by appropriate culture conditions or other strategies, including low-level enzyme supplementation, co-culture with compatible enzyme hyper producers, or engineering of the fungus to express/secrete more of the required enzymes.

Conclusions

Byssochlamys fulva AM130 could utilize both glucose and xylose and produce ethanol. It could hydrolyze alkali pretreated rice straw (PRS) to produce sugars which were fermented to ethanol under oxygen-limited condition. While endo- and exo-glucanase production was not detected, beta-glucosidase and xylanase were produced in considerable titers. This, along with the fact that the organism could

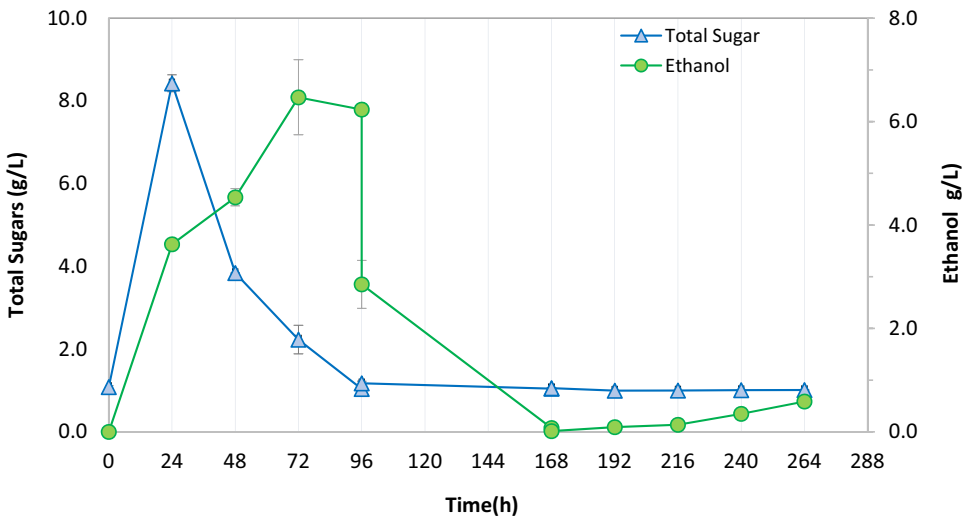

Fig. 5 Ethanol production from PRS by *Byssochlamys fulva* supplemented with cellulose. Legends: 0–96 h and 144–240 h—oxygen limited phase, 96–144 and 240–264 -aerobic phase

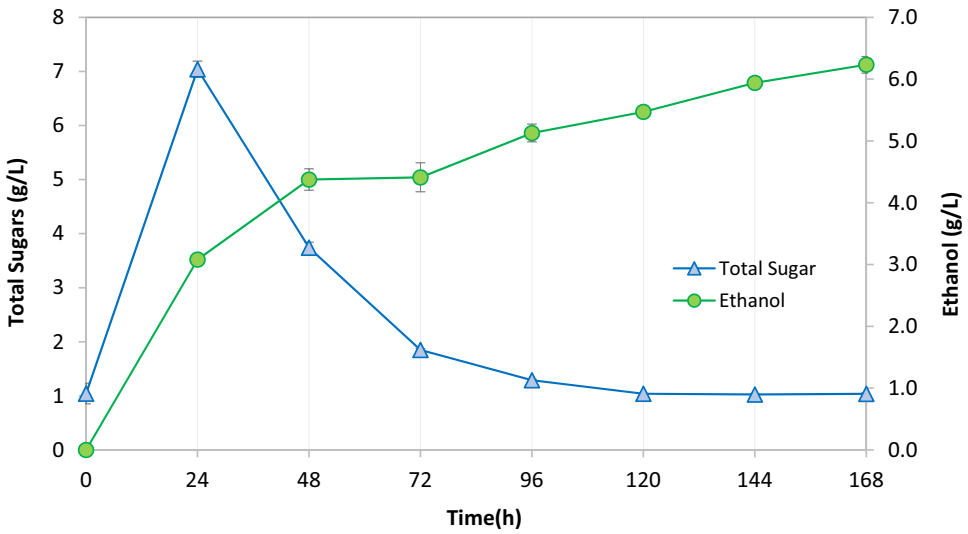

Fig. 6 Ethanol production from PRS by enzyme supplemented *Byssochlamys fulva* culture with extended anaerobic phase

directly ferment PRS to ethanol, even though at a low efficiency, indicated that the organism has the machinery for both fermentation and lignocellulose hydrolysis. Lower expression of cellulases during the fermentation phase could be due to the repression of corresponding genes in the preceding inoculum build-up phase in which glucose was used a carbon source. Cultivation strategies may be modified to address this issue and allow higher production of cellulases. The maximal ethanol yield was 11.84 g/L, but the limitation seemed to be sugar generation and not fermentation.

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