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Directed Fermentation of Water Hyacinth Biomass into bioethanol using mono, co-cultures of selected fungal strains along with *Pichia stipitis*

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Abstract: High rate of growth and easy availability of water hyacinth has made it as vibrant renewable natural carbon source for biofuel production. The present study was undertaken to screen the possibility of using water hyacinth as a substrate for bioethanol production by microbial fermentation using monocultures of *Phanerochaete chrysosporium*, *Fusarium oxysporum* individually and co-cultures of both strains with *Pichia stipitis*. Water hyacinth has high content of cellulose, hemi-cellulose, lignin and starch at levels of 18.20%, 48.70%, 3.5% and 1.85% (w/w), respectively. Direct fermentation of water hyacinth was carried out in submerged state employing the pure cultures of the spore suspensions of the fungi *Phanerochaete chrysosporium* and *Fusarium oxysporum* in separate experiments. The mixed fermentation started as a single process with a pure culture of *Phanerochaete chrysosporium* or *Fusarium oxysporum with Pichia stipitis* added at different times of fermentation. Direct fermentation of water hyacinth biomass by pure cultures of *P. chrysosporium* and *F. oxysporum* produced 12.1 g L^{-1} and 10.95 g L^{-1} ethanol respectively after 60 h fermentation. Simultaneous co-culturing of both *P. chrysosporium* and *F. oxysporum* with *Pichia stipitis* resulted in a higher ethanol production (20.17 g L^{-1}, 17.48 g L^{-1} respectively) after 60 h fermentation when compared to monoculture fermentation. Higher ethanol production was attained in the mixed culture system containing *Phanerochaete chrysosporium* and *Pichia stipitis.*

Key words: Water hyacinth biomass (WHB), Ethanol, *Phanerochaete chrysosporium, Fusarium oxysporum* and *Pichia stipitis*

Introduction: Global depletion of fossil fuels at an unprecedented rate and demand for the want of cheap energy for the world's economy, has prompted recent significant research efforts in finding viable and sustainable alternatives (Chang *et al*. 2011). Among various alternative options, conversion of abundant lignocellulosic biomasses to biofuels has gained attention. Currently, bio-ethanol production from corn and sugarcane has posed a threat to the food supply (Guragain *et al*. 2011), and the cost of these raw materials

accounts for up to 40 to 70% of the production cost (Quintero *et al*. 2008). To circumvent the use of food grade feedstock, lignocellulose rich biomass is expected to serves as potential alternative to produce low-cost bio-ethanol at a large scale (Balat, 2011). While, lignocellulosic biomasses are available from diverse sources at huge quantities and low costs, performance of microbial enzymatic saccharification depends on diverse aspects such as complex chemical compositions, structural characteristics of feedstock materials. Sugar yields from biomass enzymatic hydrolysis vary from plant to plant as a result of the differences mainly in cellulose content (Sukumaran *et al*. 2009). In recent days, screening of various lingo cellulosic substrates for biofuel has gained momentum. However, optimization of fermentative factors must be taken into consideration for large scale/ industrial scale production to meet commercial demand. This warrants special attention to the fermentation of hemicelluloses, since it is difficult to ferment by common ethanol producing organisms (Ollson and Hahn-Haherdal, 1993). Water hyacinth (*Eichhornia crassipes*) is a fast growing perennial aquatic weed invasively distributed throughout the world. This tropical plant colonizes large area of water bodies and consequently leads to series of problems like reduction of biodiversity, blockage of rivers and drainage system, depletion of dissolved oxygen, alters water chemistry that leads to severe environmental issues. In past, attempts have been geared towards the use of biological, chemical and mechanical approaches to prevent the spread/ eradication of water hyacinth. On other hand, attention has been focused on potentials of using water hyacinth for a variety of applications (Gunnarsson and Petersen, 2007). It has been reported that biomass of water hyacinth has about 48% hemicelluloses, 18% cellulose 3.5% lignin (Pothiraj et al., 2014). As the biomass productivities of WH significantly high, it has potential to serve as feedstock for bioethanol production.

Technologies for the possible conversion of WHB to bioethanol using microbial extracellular enzymes have been documented from developing countries (Idrees *et al*., 2013). *S. cerevisiae* and *Z. mobilis* are being used as candidate organisms in the large-scale production of ethanol from cellulosic biomass. These organisms are capable of utilizing hexose sugars efficiently but not the pentoses, which are the second dominant sugar source in lignocellulosic biomass (Bhattacharya *et al*., 2013). From earlier research, *P. stipitis* has been identified as an efficient strain for the conversion of pentose sugars into alcohol (Kumari *et al*., 2014)

Fermentation technologies utilizing strains of *P. stipitis* instead of the traditional yeast have been proposed (Singhal and Rai 2003., Add curent ref), as they have been shown to ferment under fully anaerobic conditions with faster specific rates of pentose sugar uptake and ethanol production as well as ethanol yields close to theoretical yield. The present study, therefore, was carried out to screen the feasibility of using hexose and pentose utilizing fungal strains for the effective conversion of water hyacinth biomass into ethanol.

Materials and Methods

Feedstock, Microorganism and Experimental design: Fresh water hyacinth biomass (WHB) was collected from a local pond at Melur, Madurai, Tamilnadu. The collected samples were washed to remove adhering dirt and then cut into small pieces (2 or 3 mm) thickness. The sample was dried at sunlight and powdered. The fungal strains of *Phanerochaete chrysosporium* and *Fusarium oxysporum* were isolated by primary selection from a naturally contaminated water hyacinth and the isolates were confirmed by their morphology and colony

characteristics (Plate 1 & 2).The isolated organisms were maintained on modified potato dextrose agar (PDA) slants at 4°C and fresh colonies were used for fermentation studies. The pure culture of *Pichia stipitis* (NCIM 3497) was procured from National Collection of Industrial Microorganisms, Pune, India. The experimental design set 1 comprised of following treatments: A - Monoculture of *Phanerochaete chrysosporium*. B- Co-culture *Phanerochaete chrysosporium* and *Pichia stipitis* C, D, E - simultaneously inoculation of *Phanerochaete chrysosporium* and *Pichia stipitis* inoculated at constant time intervals (12, 24 and 36 h).The experiment design set 2 was Monoculture of *F. oxysporum*, B – co-culture *F. oxysporum* and *P. stipitis*. C, D, E - simultaneously inoculation of *F. oxysporum* and *P. stipitis* inoculated at constant time intervals (12, 24 and 36 h)

Microbial saccharification and fermentation: Microbial saccharification and fermentation was carried out using pure and mixed cultures as described by Zabala *et al*. (1994). Erlenmeyer flasks (250 ml) containing 10% (w/v) water hyacinth biomass substrate (WHB) in 100 ml distilled water were autoclaved at 121˚C for 15 min. Late log phase culture of *P. chrysosporium or F. oxysporum* in PDA broth was used as inoculum (10%v/v) at a constant OD of 1.3. The flasks were incubated at room temperature (30±2˚C), at pH 6.0 with the agitation speed of 200rpm. Fermentation started with addition of *P. stipitis* (10% v/v) at various time intervals (12, 24 and 36h). Similarly, another set of experiment was carried out where *P. chrysosporium or F. oxysporum* as pure culture and *P. stipitis* (10% v/v) was inoculated simultaneously. Aliquots were withdrawn from the fermenting medium at regular intervals of time for the determination of ethanol, residual sugar concentration and microbial biomass. Ethanol estimation was done spectrophotometrically by potassium dichromate method (Caputi *et al.,* 1968). The microbial biomass was determined by harvesting cells by centrifugation, drying them at 70°C under vacuum to a constant weight, and expressing the dry weight as grams per 100 ml of growth medium (Doelle and Greenfield, 1985). The kinetic parameters of ethanol fermentation were determined followed by Abate *et al* (1996).

Volumetric ethanol productivity (QP) ($gl^{-1}h^{-1}$) = ethanol (gl^{-1}) /period of study (h)

Specific ethanol productivity (Qp) ($gl^{-1}h^{-1}$) = ethanol (gl^{-1})/biomass (gl^{-1}) x period of study (h)

Results and Discussion: The earlier findings on the proximate analysis of Water hyacinth biomass showed high content of cellulose, hemi-cellulose, lignin and starch at levels of 18.20%, 48.70%, 3.5% and 1.85% (w/w), respectively (Pillairaji *et al*., 2008; Pothiraj *et al.,* 2014). The cellulose and residual hemicellulose in water hyacinth (WH) were microbially hydrolyzed to fermentable oligosaccharides and monosaccharide produced on-site in simultaneous saccharification and fermentation.

Reducing Sugar The release of reducing sugars by the pure cultures of *P. chrysosporium* increased slowly to reach a peak value (34.76 gl^{-1}) at 60h fermentation. Table 1 showed that the reducing sugar levels of mixed co-culture fermentation dropped continuously reaching nearly half of the values as that of pure culture of *P. chrysosporium* at 48h fermentation. The highest increment in the reducing sugar content observed in the pure cultures of *P. chrysosporium* and *F. oxysporum* (Table 1 &2) was between 24 and 36 fermentation hours and the maximum reducing sugar (34.76 gl^{-1} and 31.13 gl^{-1}) was observed at 60 hr fermentations respectively.

Similar observations were recorded by Pothiraj *et al.* (2015) with ethanol production by a mixed culture of flocculent strains of *Z. mobilis* and *Saccharomyces* sp in the cassava substrate . Table 2 showed that the pure culture of *F. oxysporum* can ferment directly into ethanol and the pattern of change in the concentration of reducing sugars was similar to the pure cultures fermentation employing *P. chrysosporium,* but the maximum reducing sugar concentration was observed at 60 hour fermentations. The study showed a maximum ethanol productivity at 60h of fermentation due to the increased release of fermentable sugars by the co cultures.

Biomass**:** Both mono culturing and co-culturing processes showed a continuous increase in the cell biomass for both the strains up to the end of fermentation. Biomass concentration reached a maximum value of 3.12 gl^{-1} on the 60h where the yield of ethanol was 20.17gl^{-1} in the co culture fermentation of *P. chrysosporium* and *P. stipitis.* The results indicated that *P. chrysosporium* had a high growth rate during the period of glucose consumption and when *P. stipitis* began to slowly convert the reducing sugar to ethanol, the cell biomass showed moderate increase (Table 1). Inoculation of *P. stipitis* with *F. oxysporum* at 60th hr of fermentation resulted in the highest increase in biomass over the pure culture (Table 2). These values are typical of experiments reported for normal fermentations for several commercial strains grown under similar conditions (Remize 1999). Comparing the increasing rate of biomass upon the rate of fermentation revealed that (i) the higher the concentration of biomass, the quicker fermentation was completed, and (ii) that the rate of fermentation was a linear function of biomass, while fermentation time was an exponential function of biomass (Varela *et al*.2004) .

All the mixed processes reached higher value of biomass than the single fermentation process. A maximum of 3.12 g/l biomass content was obtained in the co-culture of *P. chrysosporium* and *P. stipitis* at the 60 hr phasing fermentation (Table 1). Inoculation of *P. stipitis at* 60hr after growing *F. oxysporum* resulted in the highest increase in biomass (2.64 g/l) over the mono culture (Table 2). Statistically less significant difference was observed with monocultures fermentation when compared with co-culture. Mixed cultures have been used to produce microbial biomass from other lingo cellulosic materials (Manilal *et al*., 1991).

Ethanol production: Direct fermentation of water hyacinth biomass by mono cultures of *P. chrysosporium* and *F. oxysporum* produced 12.1g/l and 10.95g/l ethanol respectively after 60 hr fermentation (Table 1 & 2). Simultaneous co-culturing of either *P. chrysosporium or F. oxysporum with Pichia stipitis* resulted in a higher ethanol production (20.17g/l, 17.48g/l respectively) at the same time. In *Phanerochaete chrysosporium with Pichia stipitis* mixed culture system, the higher ethanol production was attained in a shorter time period when compared with monoculture fermentation. The production of ethanol observed in the submerged fermentation of water hyacinth by pure cultures of *Phanerochaete chrysosporium and, Fusarim oxysporum* indicated that these organisms were capable of direct conversion of water hyacinth into ethanol. The overall production could be enhanced by co-culture rather than monoculture of test organism. Similarly direct microbial conversion of cellulosic or lignocellulosic biomass into ethanol using co-cultures had been reported by Khan and Murray(1992),Christakopoulos *et al. (*1993) and Pothiraj *et al.* (2014) reported that the ethanol concentration and yield were significantly enhanced with the mixed cultures of *Fusarium oxysporum* and *S. cerevisiae*

compared to the pure culture of the fungus in the direct conversion of sweet sorghum stalk in to ethanol and they reported 153.6% theoretical yield of ethanol while Kallel-Mhiri *et al.* (1994) got 80% theoretical yield in continuous co-cultures on whey permeate and hydrolyzed starch. *S. cerevisiae* or *Z. mobilis* utilize glucose or sucrose efficiently but their inability to utilize pentose sugars make them in appropriate candidates for refineries (Lynd *et al*., 2008). But the candidate organism *P stipitis* used in the present study showed efficient conversion of pentose sugars into alcohol.

The co-cultures of *P. chrysosporium* with *Pichia stipitis* produced maximum volumetric ethanol productivity and specific ethanol productivity (0.33±0.01 gl^{-1} $h^{-1}$and 0.107±0.01 gl^{-1} h^{-1}) respectively over the co-cultures of *Fusarium oxysporum with Pichia stipitis* (0.29±0.01 gl^{-1} $h^{-1}$and 0.11±0.001 gl^{-1} h^{-1}) on the 60hr fermentation period (Table 3). The results are clearly demonstrated that the xylose saccharification potential of *P. chrysosporium and F. oxysporum,* but the performance of both the strains in co-cultures with *P. stipitis* was significantly higher than their respective single culture.

Conclusion: The yield of ethanol recovered from treated water hyacinth through enzymatic hydrolysis and fermentation from simultaneous inoculation of co-cultures of fungal isolates with *P. stipitis* was significantly higher than that recovered through monocultures. The use of crude fungal cellulases produced on WHB in solid state fermentation would be a cost-effective approach towards enzymatic hydrolysis of identical biomass of WHB instead of using commercial cellulases. The aquatic troublemaker water hyacinth which is currently being used in waste water treatment for its unique ability to absorb heavy metal pollutants could also be utilized as abundant cheap feedstock for the production of fuel ethanol. Alcohol an eco friendly fuel, which can be produced from various renewable biological waste materials like water hyacinth can be a solution for an agricultural country like India. This study proved that water hyacinth has a potential renewable and low cost biomass for alcohol production on the commercial scale. Present cost effectiveness of respective process at commercial scale need to be standardized and the water hyacinth biomass could be a better substrate source for alcohol production.

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Table 1: Submerged fermentation of water hyacinth by mono and co-cultures of *Phanerochaete chrysosporium* and *Pichia stipitis* (Results are mean ± SE of three replicates)

Organisms	Period of Fermentations				
	Reducing sugars (gl^{-1})				
	12	24	36	48	60
A	16.12±0.8	21.28±1.7	24.51±2.1	29.18±1.6	34.76±3.1
B	48.71±27	34.28±3.4	21.75±2.4	13.12±0.6	08.43±0.3
C	-	26.28±1.3	21.18±0.9	18.15±1.1	16.42±1.2
D	-	-	25.78±1.4	23.11±1.4	17.42±1.6
E	-	-	-	25.12±2.4	17.92±1.8
Biomass (gl^{-1})					
A	1.57±0.14	1.78±0.11	2.12±0.13	2.54±0.2	2.83±0.2
B	1.73±0.15	2.18±0.21	2.31±0.19	2.73±0.14	3.12±0.15
C	-	1.93±0.15	2.35±0.14	2.68±0.25	3.08±0.29
D	-	-	2.45±0.18	2.71±0.21	2.88±0.18
E	-	-	-	2.78±0.22	2.81±0.24
Alcohol (gl^{-1})					
A	5.13±0.4	7.33±0.5	9.3±0.8	11.02±0.9	12.12±0.8
B	10.21±0.7	13.28±1.3	15.86±1.5	18.96±1.6	20.17±1.6
C	-	12.11±1.1	13.41±1.1	15.73±1.5	16.12±1.1
D	-	-	14.81±1.2	16.12±1.4	17.16±1.3
E	-	-	-	15.13±1.2	16.18±1.5

A - Monoculture of *Phanerochaete chrysosporium* .B- Co-culture *Phanerochaete chrysosporium* and *Pichia stipitis* .C, D, E,- simultaneously inoculation) of– *Phanerochaete chrysosporium* and *Pichia stipitis* inoculated at constant time intervals (12, 24 and 36 h)

Table 2: Submerged fermentation of water hyacinth waste by mono and co-cultures of *Fusarium oxysporum* and *Pichia stipitis.* (Results are mean ± SE of three replicates)

Organisms	Period of Fermentations				
	Reducing sugars (gl^{-1})				
	12	24	36	48	60
A	15.16±0.6	18.43±1.1	24.15±2.1	27.18±2.4	31.13±2.5
B	44.41±3.1	32.18±2.5	23.12±2.0	14.32±1.4	9.12±0.5
C	-	23.18±2.1	18.17±1.2	17.42±0.8	16.18±1.2
D	-	-	24.58±1.8	19.64±1.4	17.83±1.2
E	-	-	-	23.58±1.3	18.13±1.2
Cell biomass (gl^{-1})					
A	1.35±0.06	1.58±0.05	1.92±0.14	2.21±0.13	2.38±0.1
B	1.63±0.1	1.84±0.1	2.13±0.2	2.53±0.25	2.64±0.2
C	-	1.68±0.12	1.81±0.15	2.39±0.19	2.55±0.25
D	-	-	2.23±0.2	2.51±0.18	2.78±0.21
E	-	-	-	2.61±0.2	2.74±0.27
Alcohol (gl^{-1})					
A	3.93±0.25	6.12±0.45	8.15±0.44	10.12±1.1	10.95±1.2
B	9.25±0.4	11.35±0.85	14.32±1.2	15.63±1.2	17.48±1.1
C	-	9.82 ±0.5	10.53±0.7	13.15±1.0	14.48±1.2
D	-	-	11.73±0.8	13.94±0.8	14.82±1.2
E	-	-	-	14.15±1.2	15.12±1.1

A – Monoculture of *Fusarium oxysporum*, B – co-culture *Fusarium oxysporum* and *Pichia stipitis.* C, D, E,- simultaneously inoculation of *Fusarium oxysporum* and *Pichia stipitis* inoculated at constant time intervals (12, 24 and 36 h)

Table 3: Effect of microbial saccharification on the alcoholic fermentation of water hyacinth waste by mono and co-cultures of *Phanerochaete chrysosporium ,Fusarim oxysporum with Pichia stipitis. (*Results are mean ±SE of three replicates)

Organism	Fermentation time (h) (T)	Maximum ethanol 3 (gl^{-1}) (P)	Maximum biomass (gl^{-1}) (X)	Volumetric ethanol productivity (gl^{-1} h^{-1}) (QP=P/T)	Specific ethanol productivity (gl^{-1} h^{-1}) (Qp=P/XT)
P. chrysosporium	60	12.1±0.8	2.83±0.16	0.20±0.01	0.071±0.004
P. chrysosporium+ *P. stipitis.*	60	20.17±1.3	3.12±0.22	0.33±0.01	0.107±0.01
F. oxysporum	60	10.95±0.87	2.38±0.1	0.18±0.01	0.076±0.002
F. oxysporum +P. stipitis.	60	17.48±1.3	2.64±0.15	0.29±0.01	0.110±0.001

Plate1: Morphological Photomicrogaph of *Phanerochaete chrysosporium*

Plate2: Morphological Photomicrogaph of *Fusarium oxysoporum*