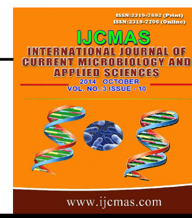

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Original Research Article

Eichhornia crassipes - a potential substrate for biofuel production

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A B S T R A C T

In the present study biofuel ethanol production was carried out from a potential substrate *Eichhornia crassipes* (water hyacinth) by separate hydrolysis and fermentation. Two fungal strains- *Aspergillus niger* local isolate and *Aspergillus niger* MTCC 2196 were used for hydrolysis of water hyacinth. Yeast strain *Sacchromyces cerevisiae* MTCC 170 was used for fermentative production of ethanol. The production was carried out in two steps-saccharification and fermentation for a period of 12 days with saccharification process of 6 days and fermentation for 6 days. Stationary fermentation method was adopted and comparative studies were performed between *A. niger* locally isolated strain and *A. niger* MTCC 2196. All experiments were carried out in triplicates and values were represented as Mean ± S.D. It was noticed that high amount of ethanol was obtained from substrate saccharified by locally isolated *A. niger* strain.

Keywords

Biofuel; ethanol; saccharification; fermentation; Saccharomyces; Aspergillus.

Introduction

The major problem of the present and future world community is global depletion of energy supply due to the continuous over utilization of resources. It is estimated that fossil fuels will be exhausted in coming decades (Cavallo, 2002). The increasing demand of ethanol for various industrial purposes such as alternative source of energy, industrial solvents, cleansing agents and preservatives have necessitated increased production of this alcohol. Ethanol production is usually accomplished by chemical synthesis of petrochemical substrates and microbial conversion of carbohydrates present in agricultural products. Owing to depleting reserves and competing industrial needs of petrochemical feedstocks, there is global emphasis in ethanol production by microbial fermentation process. Increased yield of ethanol production by microbial fermentation depends on the use of ideal microbial strain, appropriate fermentation substrate and suitable process technology (Brooks, A.A. 2008). Production of ethanol provides several advantages over gasoline like- utilization of abundant and inexpensive renewable resources, reduction in green

house gas emissions and toxic substances, macroeconomic benefits for rural community, social aspect of sustainability and pertaining in national energy security (Lin, T. 2006). Keeping in view all the above said advantages, biomass based fuel development technologies should rapidly gain momentum and the barriers imposed earlier should be removed for successfully attempting the production of ethanol at the commercial level.

Water hyacinth (*Eichhornia crassipes*) is a fresh water aquatic plant belonging to the family Pontederiaceae and is a native of Brazil and Equador region. It grows from few inches to a meter in height, floating on the surface of water (Bhattacharya A, and Kumar P. 2010). It is able to tolerate extremes in water level fluctuations, seasonal variations, nutrient availability, pH, temperature, and toxic substances (Gopal, B. 1987). It can even grow at salinity level upto 0.24% as shown in Indonesia (Kikuchi, T. et al., 1997). Shoeb and Singh (2002) reported that under favourable conditions water hyacinth can achieve a growth rate of 17.5 metric tons per hectare per day. It is an invasive species that invades fresh water habitats and is listed among with some of the worst weeds (Center et al., 1999). Water hyacinth has low lignin content, and contains high amounts of cellulose and hemicelluloses. The composition of water hyacinth is shown below in Table-1. Water hyacinth out compete almost all other species growing in their vicinity, which decreases the biodiversity (Craft et al., 2003). It devastates aquatic environment and costs billions of dollars every year in control and economic loss. Therefore presently many environmentalists are involved in the removal of water hyacinth from fresh water ecosystem. There are several ways to remove out this problematic weed from water; the best among those is to utilize the eradicated material to generate valuable products. In some places it is used as traditional medicine and even as an ornamental plant for aquariums. At present much focus is on the development of methods to produce ethanol from biomass, containing high cellulose content. As ethanol is one of the most promising replacement for fossil fuel since it is renewable, and emits less green house gases compare to gasoline. (Mete, A.M. et al., 2002). Therefore utilization of water hyacinth as a substrate for the production of ethanol will be helpful. It is advantageous over other cellulosic substrates as it contains high cellulose with low lignin content per unit volume of dry matter. Keeping in view the growing problem with water hyacinth and considering its low lignin content and high cellulose and hemicellulose content present study was undertaken with an objective to produce biofuel ethanol from water hyacinth by utilizing two fungal strains of *Aspergillus niger* for saccharification (as a substitute to cellulase enzyme) and yeast strain *Saccharomyces cerevisiae* for fermentative production of ethanol.

Materials and Methods

Collection of substrate

In present work aquatic plant water hyacinth was used as substrate. Water hyacinth (*Eichhornia crassipes*) was collected from Mir Alam Lake situated in Hyderabad Metropolis - Andhra Pradesh (Telangana region), India. The plant was harvested, collected in clean polythene bags and transferred to laboratory.

Processing of substrate

Leaves, petioles and roots were separated from the plant; roots were discarded as they

absorb heavy metal pollutants from water. Leaves and petioles were washed manually with tap water followed by distilled water to remove the waste particles, sundried and pre-treated with 1% NaOH for 2 hrs (Ali M.N. et al., 2011). After pre-treatment the plant parts were washed with water till the washed water attains neutral pH and dried in an oven for overnight. The dried leaves and petioles were grinded and filtered through 1mm sieve. The fine powder obtained was used as substrates for all saccharification and fermentation experiments.

Microorganisms used

For the present work three fungal cultures were used- two strains of *Aspergillus niger* were used for saccharification of substrate and one yeast strain *Saccharomyces cerevisiae* was used for fermentative production of ethanol.

1. *Aspergillus niger* MTCC 2196: This strain was procured from MTCC (Microbial Type Culture Collection Center & Gene Bank) Chandigarh, India. The strain was maintained on Potato Dextrose Agar medium at 30^0C. After optimum growth the culture was stored at 4^0C in refrigerator for further use.

2. *Aspergillus niger* local strain: This strain was screened and isolated from local paddy field soil. The strain was identified with the help of manuals like- Text book of Mycology by Alexopolus (1996) and Hand Book of Soil Fungi by A. Nagamani, I.K Kunwar and C. Manoharachary (2006). The strain was maintained on Potato Dextrose Agar medium at 30^0C. After optimum growth the culture was stored at 4^0C in refrigerator for further use.

3. *Saccharomyces cerevisiae* MTCC 170: This strain was procured from MTCC (Microbial Type Culture Collection Center & Gene Bank) Chandigarh, India. The strain was maintained on Yeast extract, Peptone and dextrose (YEPD) Agar medium at 30^0C.

Production of biofuel

The biofuel-ethanol was produced in two steps– a) saccharification and b) fermentation. Stationary fermentation method was adopted. For saccharification intact fungal organism *Aspergillus niger* was used as a source of cellulase enzyme. Both locally isolated *A. niger* and *A.niger* MTCC 2196 were separately used for saccharification for comparative studies. For fermentative production of ethanol *Saccharomyces cerevisiae* MTCC 170 was employed. The powdered water hyacinth was used as substrate for all the experiments. In order to optimize ethanol production the substrate was taken in three different variations as shown in table-2. Chemically defined medium (Table-3) was used in variation-3. All flasks were autoclaved at 15lbs for 15 minutes. Saccharification and fermentation studies were performed in 250 ml Erlenmeyer flasks in which 5 grams of substrate was taken in each flask (as presented in Table-2) and fermentation experiments were carried out.

Preparation of fungal inoculums for saccharification: For preparation of fungal inoculums both *A. niger* cultures were plated on PD agar plates. The plates were incubated at 30^0C for 72 hrs until the mycelium sporulates black conidia. Inoculum was produced in 250 ml

Erlenmeyer flasks containing 100 ml potato dextrose broth by transferring 2 discs of each culture from the PDA plates. The flasks were incubated for another 72 hrs at 30^0C till the mycelial mat develops. These mycelial mats were used as inoculums in saccharification experiments.

Preparation of yeast inoculum for fermentation: Yeast inoculum was prepared by inoculating loop full of *S.cerevisiae* culture in liquid YEPD medium and incubated at 30^0C for 24 hours, with agitation at 100 rpm. This suspension culture of yeast was used as inoculum for fermentation.

Saccharification of substrates: For saccharification substrate variations were autoclaved and inoculated with sporulating mycelial mats of both *Aspergillus niger* strains. Saccharification was carried out by stationary method for a period of six days at 30^0C and the process was monitored every 24 hrs for total sugars released (Miller, 1959). The fungal organism *A. niger* was selected for saccharification as it is cellulolytic in nature and can hydrolyze cellulose present in the substrates to simple sugars. Generally this step is carried out by commercially available cellulase enzyme which is very expensive. In our study an attempt was made to design an economical process by the use of intact fungal organism as a source of cellulase enzyme instead of commercially available enzyme. As *A.niger* grows on the cellulosic substrates, it hydrolyzes cellulose of the substrate and release simple sugars which can be fermented to produce ethanol.

Fermentative production of ethanol

After six days of saccharification mycelial mats of *Aspergillus niger* were removed under aseptic conditions and 10% inoculum of *Saccharomyces cerevisiae* MTCC 170 culture was added to all the flasks for fermentative production of ethanol. The process was carried out for a period of six days at 30^0C. During the fermentation process every 24 hours samples were taken for the estimation of ethanol (Caputi et al., 1968).

Statistical analysis

All experiments were performed in triplicates (n=3), values are represented as Mean ± S.D.

Results and Discussion

In the present study biofuel ethanol was produced from water hyacinth which is abundantly and cheaply available renewable cellulosic substrate by saccharification and fermentation under stationary conditions.

Ethanol production from substrates saccharified with *A.niger* MTCC 2196

Saccharification: During saccharification with *A.niger* MTCC strain, total sugars released increased from day 1 to day 4, followed by decrease upto 6th day with highest amount of sugars released on 4th day of saccharification for all substrate variations (Table-4). Among the three substrate variations, highest amount of sugars were released from substrate with chemically defined medium combination, followed by lactose combination and least were recorded with distilled water combination. With chemically defined medium combination 31.5±0.100g/100g of sugars were released on 4th day of saccharification, with lactose combination 30.5±0.100g/100g sugars were recorded and with distilled water combination a yield of 25.3±0.100g/100g of sugars released was recorded on 4th day of saccharification.

Fermentation: The results of ethanol produced from substrates saccharified with *A.niger* MTCC 2196 strain are presented in table-5. In stationary fermentation increasing trend in ethanol production was observed from day 1 to day 4. After 4th day decrease in ethanol production was observed till 6th day of fermentation. Highest amount of ethanol was produced on 4th day which is in accordance with release of total sugars. Among all the substrates highest amount of ethanol was obtained from chemically defined medium combination, followed by lactose combination and least amount of ethanol was obtained from distilled water combination indicating that the efficiency of saccharifying and fermenting enzymes on these three substrates combinations showed variations in performance.

In the case of substrate + distilled water combination lowest ethanol yield of 11.5±0.057 g/100g was recorded on 4th day of fermentation. With substrates + lactose combination high amount of ethanol was obtained (13.4±0.057g/100g), and highest ethanol was recorded from substrate + chemically defined medium combination with a yield of 13.8±0.057g/100g.

Ethanol production from substrates saccharified with *A.niger* local strain

Saccharification: With *A.niger* local strain, steady increase in release of total sugars was observed from day 1 to day 6 with highest amount of sugars recorded on 6th day. This is in variation to saccharification with *A.niger* MTCC 2196 where in highest amount of released sugars were recorded on 4th day of saccharification. The amount of sugars released upon saccharification with *A.niger* local strain was comparatively higher than that of *A.niger* MTCC strain as shown in table-6. The pattern of sugar released was similar to that of *A.niger* MTCC 2196 strain with highest amount of sugar released from chemically defined medium combination (36.4±0.351g/100g), followed by lactose combination (35.6±0.200g/100g) and least from distilled water combination (30.2±0.251g/100g.).

Fermentation: In fermentative production of ethanol from substrates saccharified by *A.niger* local strain, comparatively higher ethanol yields were obtained than the substrates saccharified by MTCC strain. The time course of ethanol production was similar to fermentation of substrates saccharified by MTCC strain with difference in yield of ethanol. With increase in time of fermentation, ethanol production increased up to 4th day. On 5th day the yield of ethanol decreased indicating that the maximum amount of sugar was consumed by 4th day. The results are shown in table-7. The trend of ethanol production followed similar pattern- highest amount of ethanol was obtained from chemically defined medium combination with a yield of 16.7±0.230g/100g, followed by lactose combination (16.2±0.057g/100g) and least ethanol was recorded with distilled water combination with a yield of 13.2±0.057 g/100g on 4th day of fermentation.

In the present work ethanol production was performed from substrate water hyacinth with saccharification process by *A. niger* and fermentative production of ethanol by *S. cerevisiae*. The substrate was chemically pretreated with 1% NaOH for a period of 2 hrs before enzyme hydrolysis to improve enzyme amenability. Lignocellulosic biomass in native form is extremely recalcitrant to enzyme saccharification, to improve enzyme amenability to holocellulose fraction a number of pretreatment methods have been designed (Wyman et al., 2005; Moiser et al., 2005 and Chandel et al., 2007). Some of the common

pretreatment methods for lignocellulosics biomass are- physical methods (pyrolysis), chemical methods (acid and alkali treatment) and enzymatic pretreatment methods. The physical and chemical pretreatment methods (in particular acid treatment) have certain disadvantages like higher temperatures are required, generation of toxic substances and giving low sugar yield. On the other hand alkali treatment is basically a delignification process, in which a significant amount of hemicelluloses are solubilized. The mechanism of action is believed to be the saponification of intermolecular ester bonds crosslinking xylan, hemicelluloses and other components (Sun et al., 2002). Alkaline pretreatment of lignocellulosics causes swelling and crystallinity, increased internal surface area, disruption of lignin structure, and separation of structural linkages between lignin and carbohydrates (Ganguly et al., 2013). Keeping in view these advantages of alkali pretreatment in the present study substrate was pretreated with NaOH.

Enzymatic saccharification is the main step in biomass to ethanol conversion. In our study an attempt was made to use the fungal culture *A. niger* as a source of cellulase enzyme in saccharification step which hydrolyzes complex cellulosic substrates by the release of extracellular cellulase enzyme and release simple sugars. The work of Chandel et al., (2007) clearly demonstrates that cost of cellulases and recovery of fermentable sugars after enzymatic saccharification are the important factors which will decide the tangible cost of biomass to ethanol process. As all these methods of saccharification adds extra cost in the production process and to the final product.

The results obtained in saccharification demonstrate that high amount of sugars were released from substrates saccharified by *A. niger* local strain and saccharification continued upto 6th day than substrates saccharified by *A. niger* MTCC strain which gave comparatively less amount of sugars and saccharification reached highest on 4th day followed by decrease in saccharification. These results are giving a clue that cellulases of local strain are efficient and performed better than cellulases of MTCC strain. Similar to saccharification, ethanol production was also highest from substrates saccharified by *A. niger* local strain than MTCC strain. Water hyacinth was also used by Magdum et al., (2012) for ethanol production by using *Pichia stipitis* NCIM 3497 where a yield of 19.2 g/l was recorded. In another report of Mukopadhayay and Chatterjee (2010) ethanol was produced from water hyacinth hydrolysate by enzymatic hydrolysis and fermentation where the yield of 8.3g/l was obtained. The results obtained in our study are better than these reports.

Ethanol production is a widely studied process for biofuel production. Different workers have studied various raw materials and different methods for ethanol production but, recently it has been observed that lignocellulosic materials are focused for fuel ethanol production as they are not a part of human food chain, do not compete with food crops and are abundantly available. Hence, we have selected cheaply and abundantly available waste biomass water hyacinth for ethanol production. There is large scope for using this waste biomass for ethanol production as this could reduce cost of cleaning water bodies which are inhabited by this deleterious water hyacinth and also producing waste to energy.

The overall results showed that cellulose utilization and cellulolytic activity and ethanol yields are low in the flasks where substrate alone is available.

Table.1 Average Composition of Water Hyacinth

S.No	Component	Percentage
1.	Lignin	10
2.	Cellulose	25
3.	Hemicellulose	35
4.	Ash	20
5.	Nitrogen	03

(Adapted from Gunnarsson and Petersen, 2007)

Table.2 Set up of saccharification and fermentation

S. No	Substrate Variation
1.	5g substrate + 100ml distilled water
2.	5g substrate + 100ml distilled water + 0.5% Lactose
3.	5g substrate + 100ml chemically defined media

Table.3 Composition of chemically defined medium (%)

Component	Percentage (%)
L-Glutamic acid	0.03
NH_4No_3	0.14
KH_2PO_4	0.2
$CaCl_2$	0.03
$MgSO_4$	0.03
Proteose peptone	0.75
$FeSO_4$	0.5
$MnSO_4$	0.16
$ZnSO_4$	0.14
Tween 80	2%

Table.4 Sugar released (g/100g) in saccharification by *A.niger* MTCC strain

Saccharification in days	*A. niger* MTCC 2196		
	D/W	Lac	CDM
Day-1	4.30± 0.100	7.23 ±0.057	8.5 0± 0.100
Day-2	10.2 ±0.100	15.3 ± 0.152	17.4 ± 0.100
Day-3	17.4± 0 .152	22.4± 0.152	24.3 ± 0.100
Day-4	**25.3 ±0.100**	**30.5 ±0.100**	**31.5 ± 0.100**
Day-5	24.4± 0.100	29.8± 0.057	31.3 ± 0.208
Day-6	23.8 ±0.057	29.4 ±0.100	29.3 ±0.100

Values are represented as Mean ± S.D.
D/W: Distilled water; Lac: Lactose; CDM: Chemically defined medium

Table.5 Ethanol produced (g/100g) by *S.cerevisiae* MTCC 170 from substrates saccharified by *A. niger* MTCC 2196

Fermentation in days	Substrate Variation		
	D/W	**Lac**	**CDM**
Day-1	3.70 ± 0.100	7.53 ± 0.057	4.8 ± 0.173
Day-2	6.26 ± 0.057	10.7 ± 0.100	7.70 ± 0.100
Day-3	8.76 ± 0.057	13.3± 0.208	11.1 ± 0.100
Day-4	**11.5± 0.057**	**13.4 ± 0.057**	**13.8 ± 0.057**
Day-5	11.0 ± 0.057	13.1± 0.057	13.6 ± 0.100
Day-6	11.0±0.100	7.53 ± 0.057	13.5± 0.057

Values are represented as Mean ± S.D.
D/W: Distilled water; Lac: Lactose; CDM: Chemically defined medium

Table.6 Sugar released (g/100g) in saccharification by *A.niger* local strain

Saccharification in days	*A. niger* Local strain		
	D/W	**Lac**	**CDM**
Day-1	4.33 ± 0.152	6.7 ± 0.100	7.6 ± 0.264
Day-2	9.73 ± 0.152	13 ±0.100	14.5± 0.305
Day-3	13.6 ±0.152	18.6± 0.152	19.5 ± 0.251
Day-4	18.4 ± 0.404	23.6 ± 0.208	25.1 ± 0.152
Day-5	24.3 ± 0.230	28.7 ±0.208	30.3 ± 0.152
Day-6	**30.2 ± 0.251**	**35.6 ±0.200**	**36.4 ± 0.351**

Values are represented as Mean ± S.D.
D/W: Distilled water; Lac: Lactose; CDM: Chemically defined medium

Table.7 Ethanol produced (g/100g) by *S.cerevisiae* MTCC 170 from substrates saccharified by *A. niger* local strain

Fermentation in days	Substrate Variation		
	D/W	**Lac**	**CDM**
Day-1	4.26 ± 0.057	5.20 ±0.100	5.93 ±0.057
Day-2	7.23 ± 0.057	8.10± 0.100	8.60 ±0.100
Day-3	10.2 ±0.100	12.1± 0.152	12.7 ± 0.100
Day-4	**13.2 ± 0.057**	**16.2 ±0.057**	**16.7 ±0.230**
Day-5	13.0 ± 0.057	16.2 ± 0.251	16.5 ± 0.288
Day-6	12.9 ± 0.057	15.7±0.100	16.5 ± 0.057

Values are represented as Mean ± S.D.
D/W: Distilled water; Lac: Lactose; CDM: Chemically defined medium

Whereas in flasks where lactose and chemically defined media are present along with the substrates showed increase in cellulolytic activity and ethanol production. Maximum cellulose utilization was observed in the flasks where chemically defined media is present along with the substrate. The results indicate that additions of chemically defined media or lactose are enhancing the cellulolytic

activity, the amount of cellulose metabolized and the total ethanol yield. These combinations gave higher ethanol yield than the substrate alone.

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