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Efficient Microorganism for Bioethanol Production from Lignocellulosic *Azolla*

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ABSTRACT

Bioethanol is a suitable renewable or alternative energy source and also the potential solution of all the problems related with the environment and energy crisis. Today ethanol derived mainly from glucose or starch sources of agriculture stock. The human demand for food, however, has yet to be met. To solve both the energy and food problems, there has been increasing interest and worldwide studies in producing bioethanol from lignocellulosic substrate. To promote bioethanol production and its uses, it is necessary to reduce the production cost by using cheap substrate viz., lignocellulosic *Azolla* and also to obtain the suitable microorganisms which provide sufficient fermentation yield. *Azolla* consisting 15.19±1.35% cellulose can be suitable substrate for bioethanol production by providing pretreatment to *Azolla* to hydrolyze the polysaccharides which released fermentable sugar. For pretreatment of *Azolla*, biological pretreatment is suitable as it is economic feasible and no toxic product formed during pretreatment. *Klebisella oxytoca* and *Saccharomyces cerevisiae*, both microorganism have the fermentation ability and can utilized for fermenting lignocellulosic substrate.

Key words: Bioethanol, micro-organisms, fermentation, lignocellulosic biomass

INTRODUCTION

In the current time, the importance of alternative energy source has become even more necessary not only due to the continuous depletion of limited fossil fuel stock but also for the safe and better environment, with an inevitable depletion of the world's energy supply, there has been an increasing worldwide interest in alternative sources of energy. 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 bioethanol at the commercial level. It is welcome to understand that the use of bioethanol as a source of energy would be more than just complementing for solar, wind and other intermittent renewable energy sources in the long run (Lin and Tanaka, 2006).

During the last two decades, advances in technology for ethanol production from biomass have been developed to the point that large-scale production will be a reality in next few years (Pandey *et al*., 2014). Ethanol production from biomass can be summarized briefly into following steps: Depolymerization of holocellulose polymer into monomeric fermentable substrate,

fermentation of depolymerized substrates and the distillation of the fermentation broth to obtain dehydrated ethanol. Bioethanol can be produced from any of the feedstocks that contain carbohydrate, fuel ethanol from lignocelluloses may also open new employment opportunities in rural areas and thus make a positive socio-economic impact. Developing ethanol as fuel, beyond its current role as fuel oxygenates will require developing lignocellulosic biomass as a feedstock because of its abundantly available and low cost.

Azolla, a pteridophyte of order salviniales consisting cellulose 15.19±1.35% (Buckingham *et al.*, 1978), can be utilized as source for bioethanol production by providing appropriate pretreatment to the *Azolla*.

Microorganisms are the only tool for bioethanol production. The microorganism must fulfill following traits for ethanol production (Dien *et al.*, 2003):

- Ethanol yield must be greater than 90% of theoretical
- Ethanol tolerance greater than 40 g L^{-1}
- Ethanol productivity rate must be 1 g L^{-1} h^{-1}
- Robust grower and simple growth requirements-inexpensive medium formulation
- Able to grow in undiluted hydrolysates-resistance to inhibitors
- Culture growth conditions retard contaminants-acidic pH or higher temperatures

The microorganism fulfilling above trait not the efficient microorganism for a particular substrate, different microorganisms behave differently for different substrate (Tiwari *et al.*, 2010; Jadhav *et al.*, 2011). The objective of this study is to find out efficient microorganism for the fermentation of fermentable sugars of biologically pretreated *Azolla*.

MATERIALS AND METHOD

MICROORGANISM

Sachharifying microorganism: *Aspergillus niger* (Zakpaa *et al.*, 2009) used to hydrolyze *Azolla* cellulose as it is able to produce the cellulase enzyme which can be tested by CMC test. The cellulase enzyme converts the cellulose in to fermentable sugar monomers which can be readily utilized by fermenting microorganism.

Fermenting microorganism: In the present study two fermenting microorganism *Saccharomyces cerevisiae* MTCC 4780 maintained in yeast peptone dextrose broth and *Klebisella oxytoca* ATCC 13182 maintained in the nutrient bacterial broth used to ferment *Azolla*.

SAMPLE PREPARATION AND PRETREATMENT

Fresh *Azolla* sample were collected, washed and dried in hot air oven at 70°C for overnight and it were grinded and allowed to biological pretreatment (Fig. 1).

For biological pretreatment *Azolla* powder 5 g was taken in 250 mL flask along with 100 mL distilled water and autoclaved at 121°C for 30 min for delignification. *Aspergillus niger* grown in potato dextrose broth for 7 days at 30°C (Eshaq *et al.*, 2011), were inoculated in the autoclaved sample for 7 days at 30°C (highest amount of sugar released at this temperature) (Eshaq *et al.*, 2011; Pandey *et al.*, 2013).

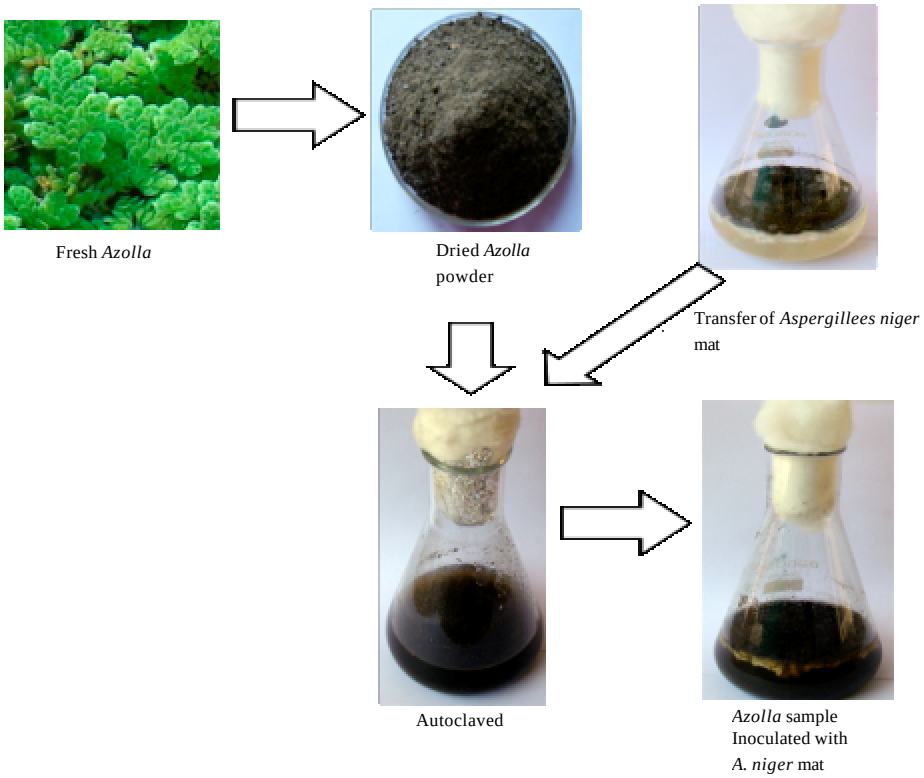


Fig. 1: Sample preparation and pretreatment of *Azolla*

FERMENTATION TECHNIQUE

Batch fermentation was carried out in 250 mL conical flask. Biologically treated *Azolla* sample were inoculated with *Klebisella oxytoca* ATCC 13182 and *Saccharomyces cerevisiae* MTCC 4780 maintained in their nutrient broth media. Quantity of inoculums was 10 mL each (Eshaq *et al*., 2011).

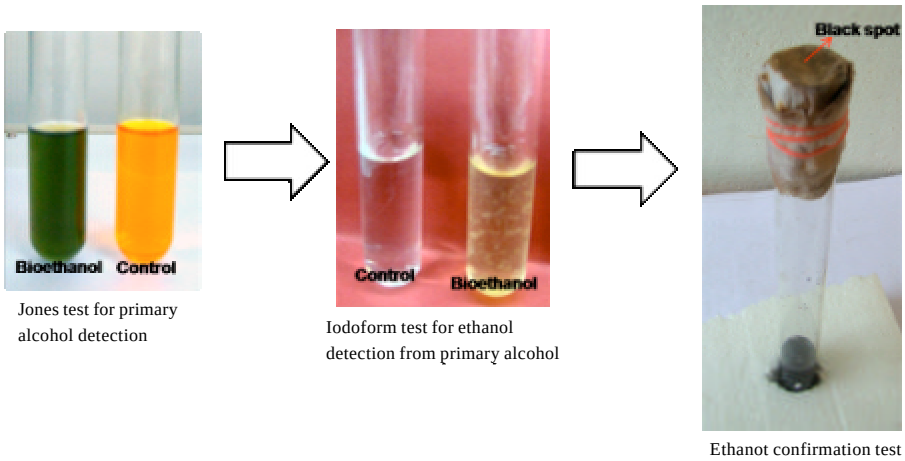
Inoculated sample were incubated at 30°C for a period of 8 days at pH 7.0 along with 1 mL of 1% solution of potassium chloride (Pandey *et al*., 2013) and the bioethanol production was estimated. *Azolla* sample was also inoculated with the consortium of both the microorganism (5 mL *Saccharomyces cerevisiae* MTCC 4780 and 5 mL *Klebisella oxytoca* ATCC 13182) for studying the synergistic activity of both the organism in fermenting *Azolla* sample.

ANALYTICAL METHOD

Sugar estimation: Released reducing sugar amount was estimated by DNS (3, 5-dinitrosalicylic acid) method using glucose as standard (Miller, 1959; Hossain *et al*., 2010).

Ethanol estimation: In fermented sample ethanol was estimated qualitatively by using Jones test (Jones, 1953), iodoform test and confirmed through black spot test using tollens' reagent. Fig. 2 shows the positive test for qualitative bioethanol estimation along with control.

Quantitative estimation of ethanol was done by using dichromate assay (Dhabekar and Chandak, 2010; Jeffery *et al*., 1989). This method uses a redox titration to find the concentration of ethanol in an aqueous solution. The ethanol is oxidized to ethanoic acid by reacting it with an



Jones test for primary alcohol detection

Iodoform test for ethanol detection from primary alcohol

Ethanot confirmation test

Fig. 2: Qualitative estimation of bioethanol

excess of potassium dichromate in acid. The amount of unreacted dichromate is then determined by adding potassium iodide which is also oxidized by the potassium dichromate forming iodine. The iodine is then titrated with a standard solution of sodium thiosulphate and the titration results are used to calculate the ethanol content of the original solution (Vogel, 1951).

RESULT AND DISCUSSION

The concentration of bioethanol in sample inoculated with *Saccharomyces cerevisiae* was 5.20%, while in sample inoculated with *Klebisella oxytoca* was 4.04% and consortium of *Saccharomyces cerevisiae* and *Klebisella oxytoca* yielded 1.98% bioethanol (Table 1).

From the above result, it was concluded that *S. cerevisiae* MTCC 4780 was more efficient in ethanol fermentation in comparison with *K. oxytoca* ATCC 13182 and consortium of *S. cerevisiae* and *K. oxytoca* (Fig. 3) studied. The *E. coli* and *K. oxytoca* are able to metabolize several sugars but are not efficient producers of ethanol (Dien *et al*., 2003). *Saccharomyces cerevisiae* is much more efficient fermenting microorganism than any other yeast (Nofemele *et al*., 2012). Potassium ion increases fermentation and it was important for the life of yeast cell under normal condition. Thus, they considered potassium as a natural activator of yeast fermentation. They also compared potassium action with sodium ion action and stated that it was weaker in action in comparison with potassium. As potassium activate the fermentation process it used as a nutrient supplement (Lasnitzki and Szorenyi, 1935). It was found that the bioethanol yield high in *S. cerevisiae* MTCC 4780 which means that potassium stimulate the fermentation process more efficiently in *S. cerevisiae* MTCC 4780 then *K. oxytoca* ATCC 13182. The result also stated that both the microorganism capable to produce bioethanol solitary but when they inoculated as combined inoculums, the yield of ethanol decline suddenly which illustrated that both microorganism are not able to produce ethanol synergistically. Different microorganism behaves differently to the substrate for fermentation. Thus, from the above, it is concluded that for efficient fermentation yield, it is necessary to use suitable microorganism along with the optimatization of different fermentation parameters.

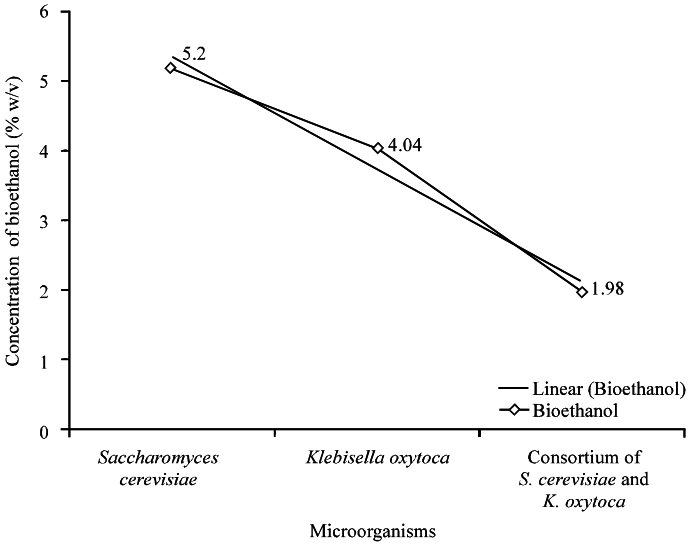


Fig. 3: Bioethanol production from biologically treated *Azolla* using different microorganism

Table 1: Effects of different Microorganisms on bioethanol production from biologically treated *Azolla*

Micro-organism	Concentration of bioethanol (in % w/v)	Remark
S. cerevisiae MTCC 4780	5.20	Maximum
K. oxytoca ATCC 13182	4.04	
Consortium of *S. cerevisiae* and *K. oxytoca*	1.98	Minimum

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