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Production of Bioethanol from Papaya and Pineapple Wastes using Marine Associated Microorganisms

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In the era of declining fossil fuel resources, the World is in urgent need to look into the possibilities of alternate energies which shall sustain the energy resources. Among the alternative energies, biofuels such as bioethanol, biodiesel and methane are advantageous. In this study, an attempt was made to produce bio-ethanol by marine fungi in fermentation process with the use of fruit wastes (papaya and pine apple) as substrates. A total of 19 marine fungi were isolated from various marine and marine related specimens such as sea surface water, rotten woods found along beaches, marine sediment sample and rhizosphere soil from coastal sand dunes. The isolates were studied for their capability to produce enzymes to help in fermentation processes like pectinase, amylase and cellulase. Amongst these isolates, strain AMETF018 was selected because of its potential in producing pectinase, amylase and cellulase. The fungus was grown in liquid culture and mycelia biomass was immobized with calcium alginate. A total of 12 set of fermentation experiments were carried out by immobilized fungus and commercial baker's yeast *Sacchromyces cerevisiae* using papaya and pineapple wastes as substrates for ethanol production. The combination of pineapple fruit waste, immobilized marine fungus AMETF018 and fermentative yeast *S. cerevisiae*, was found to be the best suited for higher production of ethanol. This study thus concludes that marine fungi in combination with yeast could be a potential source for the effective utilization of fruit wastes as biofuel.

Key words: Fruit wastes, marine fungi, enzymes, bioethanol, fermentation.

Rapid increase in energy consumption and depletion of fossil fuel has lead to an extensive research in the field of alternative fuel. In this context, bioethanol has been reported as an alternative fuel because it is renewable, non toxic and biodegradable (Harun *et al*., 2011; Borines *et al*., 2013). Bioethanol can be derived from biomass with many different varieties of feed stocks such as corn, sugarcane, wood and fruits wastes which are easily accessible and reliable and can help to clean the environment from the wastes (Patni *et al*., 2013; Suryaningsih *et al*., 2014). According to Berg (2001), fuel ethanol, besides its environmental value, is and will remain first and foremost an instrument to support farmers, as they will profit

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from fuel ethanol programmes. Biofuels give more benefit since it comes from renewable resources. It's sustainability reduces greenhouse gas emission, regional development, social structure, agriculture and security supply (Demirbas 2006). However, to date most of the bioethanol is being produced by using food crops which may surely affect the food security in this population exploding era.

Waste is an inheritable consequence of the food industry (Rodriguez *et al*., 2003). Food industry produces large volumes of wastes, both solids and liquid, resulting from the production, preparation and consumption of food. These wastes pose increasing disposal and potential severe pollution problems and represent a loss of valuable biomass and nutrients. The wastes contain valuable components such as: sucrose, glucose, fructose and other Nutrients (Sasaki *et al*. 1991; David *et al*., 2008). Various studies have suggested utilization of these wastes for the production of single cell protein, ethanol, enzymes etc. (Van Dyk *et al*., 2013). Ethanol fermentation depends on the nature of the substrate used. Therefore, it is important to select efficient raw materials for ethanol fermentation (Baptista *et al*., 2006). Fruit pulp wastes after extracting juices are one of the major by products of food processing industries. By-products of plant food processing represent a major disposal problem for the industry concerned, but they are also promising sources of biomaterials (Schieber, 2001). These biomaterials can be used as substartes for bioethanol production. Hydrolysing enzymes ferment the complex sugars to reducing sugars and then to high concentrations of ethanol. It is also being made from a variety of agricultural by-products such as grain, fruit juices, fruit extracts, whey, sulfite waste liquor and molasses (Nigam *et al*., 1998). In the early 1970s, the oil crisis generated interest in using cellulose as a chemical and energy resource. Cellulose is a secondary nutrient source for fungi Eriksson *et al*. 1990). One promising approach was to hydrolyse the cellulose to glucose with fungal enzymes and then to ferment the glucose to ethanol which could be used as a liquid fuel (Mandels*et al.,* 1974). A variety of microbes are shown to produce ethanol (Soccol *et al*., 2010; Dias *et al*., 2011). The role of the microorganisms (bacteria and fungi) in the process of fermentation showed that waste pineapple, banana and pawpaw fruits contain a fermentable material which was evident from the increase in the acidity of the fermenting juice (Ogunjobi*et al*., 2005). A number of organisms including fungi, yeast and bacteria have been screened for ethanol fermentation. Extensive studies have been carried out on the fermentation process of ethanol by these organisms, especially through yeast cells (Bajaj *et al*., 2001). Ethanol is produced by fermentation: when certain species of yeast (notably *Saccharomyces cerevisiae*) metabolize sugar in the absence of oxygen, they produce ethanol and carbon dioxide. (Anxo*et al*., 2008). Immobilization of microbes has been reported to enhance the production of ethanol (Mariam *et al*., 2009). Hence in the present investigation we have attempted to produce ethanol from pineapple and papaya waste using immobilized fungi and commercial yeast, *Saccharomyces cerevisiae.*

MATERIALS AND METHODS

Isolation of marine associated fungi from coastal samples

Sea surface water, wood wastes, coastal sediments and and rhizosphere soil of plants (*Ipomoea* sp. and *Spinifex* sp.) were collected from the coastal area of Uthandi, Chennai (Lattitude 12.872854, Longitude 80.250851), India during March 2012. Immediately after collection, samples were brought to the laboratory in sterile polythene bags. Marine associated fungi were isolated using serial dilution plating technique in potato dextrose agar prepared in 50% of seawater (Muthezhilan, 2008).

Screening the isolated fungi for the production enzyme activity

Screening of enzyme activity by marine fungi was tested by qualitative plate assay. Minimal salts medium (K_2HPO_4 25.6 g/L; KH2PO4 6 g/L ; NaCl 1 g/L ; NH4Cl 2 g/L; Agar 20 g/L; pH 6.5) supplemented with different substrates for the test enzymes was prepared separately and mycelia discs (6 mm diameter) from actively growing regions of individual marine fungi was inoculated and incubated for 7 days. Pectin (0.3%), CM Cellulose (0.3%) and soluble starch (0.3%) were used as substrates for pectinase, cellulose and amylase respectively. Pectinase and CM Cellulase activity was visualized as a zone of clearance

against red background when the plates were stained with congo red (0.3% for 15 minutes) followed by destaining with 1N NaCl (for 15 minutes). Amylase activity was visualized as a zone of clearance against dark blue background when the plates were flooded with Gram's Iodine. Zone of clearance of all the enzyme activities were recorded.

Immobilization of fungal mycelia using calcium alginate

The selected marine fungus AMETF18 which produced enzyme activities for pectinase, cellulose and amylase was chosen for further studies. The fungus was grown on potato dextrose broth and incubated for 3 to 5 days. After incubation, the liquid cultures were centrifuged at 3000 rpm at 10°C for 8 min and the pellet was suspended with a previously autoclaved solution of sodium alginate. The mixture was added drop wise into an autoclaved solution of calcium chloride to form beads. The beads were left in the gelling solution for 5 min before being harvested and rinsed with sterilized physiological saline solution. It was then chilled overnight at 4°C.

Immobilization of commercial yeast (dry and grape yeast) - calcium alginate

Two types of yeast cultures were used the immobilization. Dry Yeast (Y1) was obtained as a granulated formulation of *Saccharomyces cerevisea*. Fresh yeast (Y2) was isolated freshly from the grapes. They were grown on malt yeast glucose peptone broth and incubated for 4 days. 2 g sodium alginate was mixed in 100 ml of boiling water with constant stirring on magnetic stirrer. After cooling sodium alginate solution, 2 g of yeast cells were added to the slurry under stirring conditions for even dispersal. The slurry solution, with yeast biomass was dispersed drop wise into 2% chilled $CaCl_2$ solution. Spherical beads were formed which were washed with 0.2% chilled $CaCl_2$ solution and stored at 4°C for further use to carry out fermentation.

Fermentation of papaya and pineapple wastes with immobilized yeast and fungi

Fermentation was carried out in 100 ml Erlenmeyer flasks using immobilized beads. Briefly, 50 ml of sterile distilled water with 10 g pine apple or papaya waste was added in each flask according to the treatment. 500 immobilized fungal beads or immobilized yeast beads or both were added as per the treatment. It was then mixed properly and maintained at 30°C for about 4 weeks. After incubation, the beads were separated for further use and the fermented medium was used for estimation of ethanol contents using a simple formula as given below

Alcohol by volume (ABV)=
[initial gravity – final gravity] / 0.0075

RESULTS AND DISCUSSION

The unique physico-chemical properties of the marine environment are likely to have conferred marine fungi with special physiological adaptations that could be exploited in Biotechnology. Fungi occurring in decomposing plant organic material or detritus in the sea have been shown to be source of several wood-degrading enzymes of importance in paper and pulp industries and bioremediation (Raghukumar, 2008). Agro-industrial wastes are generated during the industrial processing of agricultural products. Although the production of bioethanol offers many benefits, more research is needed in the aspects like feedstock preparation, fermentation technology modification, etc., to make bioethanol more economically viable (Bhatia *et al*., 2012). In this context, we have undertaken a preliminary study on the suitability of marine associated fungi for the production of ethanol using fruit wastes such as papaya and pineapple. These marine associated fungi are having the ability to produce enzymes such as pectinase, cellualse and amylase. These enzymes are having inherent potential to convert complex biomass in to simpler and fermentable carbohydrates or similar biomolecules. Such converted biomaterials then be fermented with fermenting yeasts to produce ethanol.

A total of nineteen different fungal isolates were isolated from four different samples such as sea surface water, wood wastes, coastal sediments and rhizosphere soil of plants *Ipomoea* sp and *Spinifex* sp collected from Uthandi, Chennai, India and named as AMETF1 to AMETF19. In the present study, all the 19 strains were subjected to screen for the production of different hydrolytic enzymes. Out of 19 strains, twelve strains were able to show cellulolytic activity. Among then a strain AMETF6 produced maximum cellulose activity (2.7 cm zone of clearance). Further, 11

strains showed pectinolytic activity using pectin as substrate and AMETF7 showed the highest pectinase activity (2.7 cm zone of clearance) and three strains showed amylolytic activity. Interestingly, a strain AMETF18 strains produced all the three enzymes, hence, it was selected as potential strain for further study (Table 1). In general, biomass to ethanol bioconversion process consists of several steps, including pretreatment of biomass, enzymatic hydrolysis, fermentation and product recovery. Enzymatic hydrolysis is the key to cost-effective ethanol production from lignocellulosic substrates in the long run, as it is very mild process, gives potentially high yields, and the maintenance costs are low compared to acid or alkaline hydrolysis (Kuhad *et al*. 1997; Saini *et al*., 2014). Cellulose and pectin are the most dominantly present biomolecules in the agricultural wastes such as fruit wastes. Hence organisms producing cellulase and pectinase have the great potential for the bioconversion of complex agriwastes in to fermentable sugars. The selected strain AMETF18 has the potential to produce pectinase, cellulase and amylase.

Pineapple waste is rich in cellulose, hemicelluloses, sugar and other carbohydrates. Several authors has used pineapple waste for the production of ethanol (Alan *et al*., 1987; Nigam, 1999; Tanaka *et al*., 1999; Chanprasartsuk *et al*., 2010). Recently, pineapple peel has been reported to yield ethanol (9.69 g/L) after 72 h of fermentaion by *S. cerevisiae* (Choonut *et al*., 2014). Protease produced during maturation of pineapple fruit can cause lysis of microbial cell wall (Singleton and Gortner, 1965; Uhlig and Linsmaier-Bednar, 1998), which in turn can damage the yeast cells. In this regard, immbolization can be suggested as an efficient methods in fermentation (Kaetsu *et al*., 1987; Agudelo and Penuela, 2010). Sharma and Ogbeide, (1982) have suggested papaya as a renewable energy resource for the production of alcohol using yeast *Sacchromyces carlsbengensis*. Lee *et al*. (2010) have investigated the formation of compounds such as fatty acids, alcohols, aldehydes and esters in papaya juice fermented by a mixed culture of *Saccharomyces cerevisiae* and *Williopsis saturnus.* Papaya juice fermentation with yeast *W. saturnus* var *mrakii* NCYC 2251 was carried out by Lee *et al*. (2011). Nigam, 2000 has used immobilized yeast cells to produce ethanol production from pineapple cannery waste.

Table 1. Screening of Marine fungi for the production of fermentative enzymes

S. No	Strain No	Production of Enzyme Activity/Zone of Lysis (cm)		
		Cellulase	Pectinase	Amylase
1	AMETF1	1.2 ± 0.1	-	1.1 ± 0.1
2	AMETF2	1.1 ± 0.1	-	1.4 ± 0.05
3	AMETF3	-	2.4 ± 0.1	-
4	AMETF4	-	-	-
5	AMETF5	1.4 ± 0.05	2.5 ± 0.1	-
6	AMETF6	2.7 ± 0.05	2.5 ± 0.1	-
7	AMETF7	-	2.7 ± 0.1	-
8	AMETF8	-	-	-
9	AMETF9	1.6 ± 0.1	-	-
10	AMETF10	1 ± 0.05	-	-
11	AMETF11	1.4 ± 0.1	-	-
12	AMETF12	1.1 ± 0.05	-	-
13	AMETF13	1.1 ± 0.1	1.3 ± 0.1	-
14	AMETF14	-	2.1 ± 0.1	-
15	AMETF15	-	2.1 ± 0.3	-
16	AMETF16	2 ± 0.2	2 ± 0.1	-
17	AMETF17	-	1.4 ± 0.2	-
18	AMETF18	1 ± 0.04	1.3 ± 0.1	1.3 ± 0.1
19	AMETF19	1 ± 0.04	1.3 ± 0.1	-

In this context, we have immobilized both selected marine fungus and fermentative yeasts using calcium alginate as matrix and got immobilized beads with desirable qualities. Simultaneous saccharification and fermentation was carried out with 12 different combinations of treatments. The application of simultaneous saccharification and fermentation (SSF) for the conversion of lignocellulosics to alcohol would result in a more cost-effective process. Aptly, lignocellulosic substrates were examined in SSF experiments for the production of ethanol using two yeast strains (a commercially available baker's yeast and a thermotolerant *Kluyveromyces marxianus*) (Kádár *et al*., 2004). In our study, high level of ethanol yield was obtained only with the combined fermentation of both the waste (papaya and pineapple) with both selected marine fungus AMETF18 and commercial baker's yeast and the results of fermentation experiment was provided in Table 2. The highest ethanol production i.e. alcohol by volume was observed in the Fungus+ Dry yeast + Pine apple waste treatment (7.5%) followed by Fungus+ Fresh yeast + Papaya waste treatment which yield 6.5% alcohol. Previously, studies were carried out on the ethanol production by free and immobilized *Saccharomyces cerevisiae* GC-IIB31 under stationary culture. Cane molasses in different concentration was used as sugar source for maximum conversion of reducing sugar into ethanol (Mariam *et al*., 2009). Thus the present study indicates the potential of selected marine fungus AMETF18 along with bakers yeast for simultaneous saccharification and alcohol fermentation in laboratory conditions. However, these studies are further to be refined by experiments with bioreactors.

Table 2. Estimation of ethanol content in different fermentation experiments

Fermentation treatment	Alcohol by volume (%)
Pine apple waste	0
Papaya waste	0
Fungus+ Dry yeast + Pine apple waste	7.5 ± 0.04
Fungus+ Fresh yeast + Papaya waste	6.5 ± 0.04
Fungus + Fresh yeast + Pine apple waste	5.6 ± 0.04
Fungus + Dry yeast + Papaya waste	5.5 ± 0.04
Fungus + Pine apple waste	3.4 ± 0.04
Fungus + Papaya waste	2.1 ± 0.05
Dry yeast + Pine apple waste	2.1 ± 0.08
Dry yeast + Papaya waste	2.3 ± 0.05
Fresh yeast + Pine apple waste	1.6 ± 0.05
Fresh yeast + Papaya waste	1.6 ± 0.1

Fig. 1. Pure cultures of Marine associated fungi

Fig. 2. Fermentation of papaya and pineapple wastes using different combinations of immobilized marine fungi and yeast

CONCLUSION

In our study pineapple and papaya wastes were used as a substrate by fungus to produce ethanol with a maximum yield of 7.5 % v/v. In conclusion, our study suggests the possible use of pineapple and papaya wastes with immobilized marine fungus and bakers years. Nevertheless, more studies are required to identify, isolate, and study potential fungus for the production of bioethanol from these wastes.

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