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# Bio-ethanol production by marine yeasts isolated from coastal mangrove sediment

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**Abstract:** This study analyzed ethanol production by 10 marine strains of yeasts, isolated from mangrove sediments. Of the species tested, Pichia salcaria exhibited the maximum ethanol production of 12.3±3.1 g/l. This was further studied in the culture filtrate supplemented with different concentrations (0.01, 0.1, 1, 2 and 4% w/v) of sawdust as substrate that was pre-processed with phosphoric acid, and then incubated for different durations (24,48,72, 96 and 120 h). The ethanol production by P. salcaria was found maximum (26.2±8.9 g/l) in 72 hours of fermentation in the culture broth supplemented with 2% sawdust that was preprocessed with dilute phosphoric acid. This condition of fermentation was found coincided with high activity cell ase in the cul ure broth, which also revealed a single prominent protein band of 68 kDa molecular weight, similar to cellulase. The study proved the efficiency of marine P. salcaria in bioethanol production.

**Keywords:** Bioethanol, Mangroves, Yeasts, *Pichia salicaria,* Cellulase

## INTRODUCTION

Cellulose is the most abundant form of organic carbon, synthesized by plants and it is a linear polymer, composed of glucose sub-units held together by β-1,4-glucosidic bonds. Cellulosic materials are renewable natural biological resources which can be used for the production of biofuels (Zhang et al. 2007). Various industries utilize huge volumes of cellulosic wastes which provide a low-cost and sustainable resource for production of ethanol (Das and Singh 2004). Cellulose can be effectively hydrolyzed and depolymerized in to fermentable sugars by the enzyme cellulase. This enzyme is also used for biological conversion of plant biomass into fuels and high value chemicals, which finds a wide range of applications, especially in food, animal feed, textile, fuel and chemical industries (Mandels 1985). The conversion of cellulose into ethanol by means of cellulase is the recent drift in biofuel industries. There are mainly two processes involved in the conversion: hydrolysis of cellulose in the lignocellulosic biomass to produce reducing sugars; and, fermentation of the sugars to ethanol (Sun and Cheng 2004). The bioethanol made from renewable resource provides environmental protection by reducing global warming, economic development, and energy security in the present context of raising emissions of green house gases with the rapidly exhausting oil resources.

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A number of microorganisms are capable of producing extracellular cellulase enzyme (Kirk *et al.* 2002). Yeasts such as *Pichia stipitis*, *Candida shehatae* and *Pachysolan tannophilus* have the ability to use both C5 and C6 sugars (Agbogbo and Coward-Kelly 2008). However, ethanol production from sugars derived from starch and sucrose has been commercially dominated by the yeast *Sacchromyces cerevisae* (Lin and Tanaka 2006; Tian et al. 2009).

Even though, yeasts are known to be important candidate for fermentation, its application in ethanol production utilizing cellulosic materials has not received proper attention. All these works are also confined to only terrestrial strains of yeasts but not their marine counterparts. Hence, the present study was conducted to analyse ethanol production by yeasts of marine origin on saw dust as the substrate.

## MATERIALS AND METHODS

### Chemicals

All analytical reagents and media components were purchased from Hi-media (Mumbai, India).

#### *Marine yeast species*

Ten species: *Candida albicans, C. tropicals, Debaryomyces hansenii, Geotrichum* sp., *Pichia capsulata, Pichia fermentans, Pichia salicaria, Rhodotorula minuta, Cryptococcus dimennae* and *Yarrowia lipolylica*, isolated from sediments of the mangrove forests in southeast coast of India (Lat. 11°27' N; Long. 79°47'E) were used in the present study after identification. The yeast strains were deposited in the microbial collection centre of the CAS in Marine biology, Annamalai Univerisity.

### Preparation of saw dust

The saw dust obtained from a wood mill was processed mechanically to reduce the length of fibers, prior to

pretreatment. After removing the minor impurities by means of washing, they were oven-dried at 60ºC for overnight. The saw dust then obtained was pretreated (Galbe et al. 2005). The saw dust weighing 50 g was washed to remove the wood residues, then treated with 1L of 4% sodium hydroxide, and autoclaved at $121^0$C for 30 minutes. The material recovered by filtration was washed with distilled water and dried at $65^0$C to a constant weight. One portion of the powder obtained before the successive washes was neutralized with phosphoric acid and filtered. Same volume of distilled water was added to the powder thus obtained, and heated at $121^0$C for 30 minutes. The suspension was filtered and the solid materials were dried at $65^0$C to constant weight.

After drying, one sample was kept as unprocessed without any treatment. Another one was processed with distilled water; while the other two were treated with 0.8% diluted and concentrated phosphoric acid. All the processed sawdusts were supplemented at different concentrations, 0.01, 0.1, 1.0, 2.0 and 4.0 g per 100 ml of yeast culture medium. The ethanol concentration was estimated during 24, 48, 72, 96 and 120 h of incubation by using a gas chromatography.

**Production of bioethanol**

The production of bioethanol was done using marine yeast fermentation by following the method outlined by Caputi et al. (1968). In this method, 1 ml of the yeast was inoculated in Yeast Malt Broth (Dextrose-5.0 g, Peptone-5.0 g, Yeast extract-3.0 g and Malt extract-3.0 g in 1L 50% seawater). The fermentation was carried out in 500 mL Erlenmeyer flasks using 100 mL of medium. After tightly closing, the flask was kept for fermentation at 28°C for 120 hours on a shaker at 120 rpm. The level of ethanol in all the flasks was estimated at every 24 h time interval of incubation.

**Estimation of bioethanol by using gas chromatography**

Ethanol concentration in the samples was estimated using a Hewlett Packard 5890 Series II gas chromatography with nitrogen as a carrier gas. The temperature of the injection port, oven and detection ports were 250,120, and $250^0$C, respectively. For the analysis, 2 mL of liquid samples was withdrawn from the fermentation broth in gas tight syringes and then injected into the gas chromatography. The ethanol concentration was determined by using ethanol standard plot and is expressed in percentage of ethanol.

**Cellulase assay of the fermentation broth**

To confirm the role of cellulase enzyme in ethanol production, carboxy methyl cellulase activity was assayed using 0.1 ml of water, 2.0 ml of 1% (w/v) carboxy methyl cellulose (CMC) in 0.1 M acetate buffer (*p*H 5.0), and one ml of the fermentation broth by incubating at 50°C for 20 min. The amount of reducing sugar formed was measured by the method as described by Miller (1987). One unit of CMC activity was defined as the amount of enzyme that liberated one µmol equivalent of glucose under the assay condition. The cellulase activity was estimated after 24, 48, 72, 96 and 120 h of incubation.

**PAGE analysis of the fermentation broth**

The fermentation broth was precipitated with solid ammonium sulphate at 80% saturation. The precipitate obtained was collected by centrifugation at 15,000 × g for 20 min and dissolved in 0.2 M acetic acid–sodium acetate buffer with 1.4mM of 2-mercaptoethanol. This extract was dialyzed overnight against the same acetate-buffer. The dialyzed solution was clarified by centrifugation and 2 ml of the supernatant was mixed with 2 ml of Laemmli's buffer and boiled for 5 min and protein analysis was carried out by using 10% SDS-PAGE, according to Laemmli's method (1970). Samples were diluted at 1:5 with Laemmli sample buffer. Protein in the gel was stained with Coomassie blue R-250 (Brillant blue R) and its molecular weight was estimated by comparing with standard proteins of 10-250 kDa molecular weights.

**Statistical analysis**

Statistical analysis was made by analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT). Results are expressed as mean ± standard deviation from triplicates in each group. The $P$ values < 0.05 were considered as significant.

## RESULTS

***Screening of marine yeasts for alcohol production***

The ethanol production varied with yeast species. *Pichia salcaria* showed the maximum ethanol production of 12.3g/l while *Candida albicans* exhibited the minimum of 1.7 g/l (Table 1).

**Effect of sawdust treatment on ethanol production**

*Pichia salicaria* produced ethanol on both unprocessed and processed cellulosic material (Table 2). The maximum production of 26.2g/l was recorded in the sawdust processed with 0.8% dilute phosphoric acid, while the minimum (7.2g/l) was noted in the sawdust processed with concentrated phosphoric acid.

**Optimization of sawdust concentration for ethanol production using marine yeast**

The ethanol production varied significantly between concentrations of sawdust. The sawdust processed with 0.8% dilute phosphoric acid exhibited an increase in ethanol production with increasing concentrations of sawdust (Fig. 1). The maximum production of 26.2 g/l ethanol was observed when 2% of sawdust was added. However, the sawdust processed with concentrated phosphoric acid exhibited only minimum levels of ethanol at all concentrations.

**Optimization of time interval for ethanol production using marine yeast**

The ethanol production by *Pichia salcaria* increased with time of incubation and it was the maximum at 72 h of incubation in all the treated sawdust. There was no further increase in the production (Fig. 2).

**Cellulase activity in the fermented broth**

Cellulase activity was noticed in the fermented broth of *Pichia salcaria* supplemented with 2% sawdust pre-processed with dilute phosphoric acid, incubated for 0, 24, 48, 72, 96 and 120 h. The cellulase activity varied significantly between the time of incubation, but the activity was the maximum at

72 h of incubation (Table 3).

**Partial purification of protein of culture broth of Pichia salicaria**

The molecular weight estimated by SDS-PAGE of the protein revealed multiple bands protein band (Fig.3). After 24 and 48h, two prominent bands with the molecular weights of 68, and 150 kDa were observed, while after 72h, only a single prominent band of 68kDa was revealed. However, no prominent bands were noted after 96 and 120h.

Table 1. Ethanol production in culture filtrates of marine yeasts after 120 hours of incubation

| S. No. | Name of marine yeast spices | Ethanol production (g/l) |
|---|---|---|
| 1. | *Candida albicans* | 1.7±0.1[a] |
| 2. | *Candida tropicalis* | 2.2±0.5[a] |
| 3. | *Debaryomyces hansenii* | 2.0±0.5[a] |
| 4. | *Geotrichum.sp* | 5.3±0.9[b] |
| 5. | *Pichia capsulata* | 12.3±0.8[b] |
| 6. | *Pichia fermentans* | 8.1±0.6[c] |
| 7. | *Pichia salicaria* | 7.3±0.1[d] |
| 8. | *Rhodotorula minuta* | 4.2±0.8[b] |
| 9. | *Cryptococcus dimennaea* | 4.5±0.9[b] |
| 10. | *Yarrowia lipolytica* | 7.6±0.6[c] |

Each value is mean ± standard deviation for 3 replicates in each group
Values not sharing a common superscript differ significantly at $P < 0.05$ (DMRT)

Table 2. Effect of various types of sawdust processing on ethanol production

| S. No. | Type of processing | Ethanol production (g/l) |
|---|---|---|
| 1. | Unprocessed | 12.8±3.7[a] |
| 2. | Processed with distilled water | 14.7±5.9[b] |
| 3. | Dilute phosphoric acid | 26.2±8.9[c] |
| 4. | Concentrated phosphoric acid | 17.2±3.1[a] |

Each value is mean ± standard deviation for 3 replicates in each group
Values not sharing a common superscript differ significantly at $P < 0.05$ (DMRT)

Table 3. Cellulase activity in the fermented broth of *Pichia salicaria* supplimented with 2 % of sawdust processed with dilute Phosphoric acid during different time intervals

| Time intervals | Ethanol production ( g/l)) | Cellulase Activity (U*/ml) mean ±SD |
|---|---|---|
| 24h | 15.2.9± 1.3[a] | 10.2 ± 1.7 [a] |
| 48h | 20.4± 1.9[b] | 13.3± 2.9 [b] |
| 72h | 26.2± 2.9[c] | 14.1 ± 2.3 [c] |
| 96h | 22.2± 2.4[d] | 13.5 ± 1.9 [d] |
| 120h | 21.1± 2.1[e] | 10.4 ± 2.1 [c] |

* One unit of cellulase activity was defined as the amount of enzyme that liberates 1 µmol equivalent of glucose under the assay conditions.
Values are mean ± standard error from 3 replicates in each group
Values not sharing a common superscript letter differ significantly at P < 0.05 (DMRT)

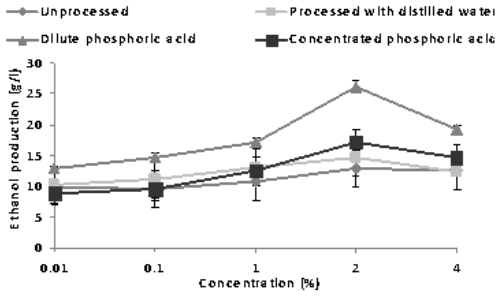


Fig.1. Ethanol production by *Pichia salicaria* culture broth supplemented with various concentrations of processed and or unprocessed sawdust

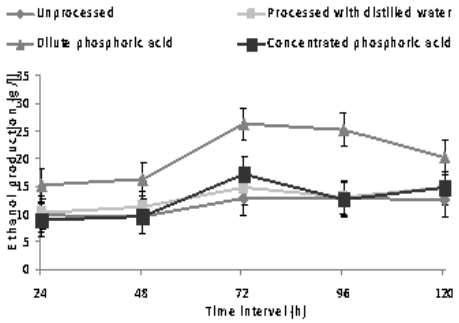


Fig.2. Ethanol production by *Pichia salicaria* culture broth supplemented with sawdust incubated at different time intervals

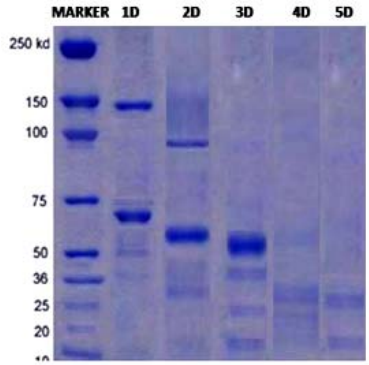


Fig 3. SDS-PAGE patterns of purified protein of fermentation broth of *Pichia salicaria* during different days of incubation (SDS-PAGE carried out using 10% polyacrylamide gel containing 0.1%SDS, and stained with 0.1% Comassie brilliant blue R- 250) after electrophoresis.
Lane: MARKER-molecular weight marker
Lane: 1D, 2D, 3D, 4D and 5D - purified protein of cell filtrate of *Pichia salicaria* during day 1, 2 3, 4 and 5 respectively.

## DISCUSSION

Many microorganisms such as bacteria, fungi and yeasts are employed in ethanol fermentation processes. Yeasts are advantageous over other microbes for commercial fermentation due to their larger sizes, thicker cell walls, better growth at low pH, less stringent nutritional requirements, and greater resistance to contamination (Jeffries. 2006). There are many studies on alcohol production from yeast fermentation (Lark *et al.* 1997). However, such studies are restricted to yeasts of terrestrial origin. The present study proved that ten marine yeasts species were capable of producing ethanol with the maximum production of 13.2 g/l by *Pichia salcaria* (Table 1). When the sawdust was processed with 0.8% diluted phosphoric acid the ethanol production was found maximum of 26.2 g/l within 72 hours. Hydrolysis of cellulosic materials by concentrated sulfuric or phosphoric acids is a relatively old process. Reaction times are typically much longer for concentrated acids than for dilute ones. In general, concentrated phosphoric acid is used followed by a dilution with water to dissolve and hydrolyze or convert the substrate into sugar. This process provides a complete and rapid conversion of cellulose to glucose and hemicelluloses to 5-carbon sugars with little degradation. The critical factors needed to make this process economically viable are to optimize sugar recovery and cost effectively recovers the acid for recycling. Earlier studies conducted using terrestrial yeast, mostly *Saccharomyces cerevisiae* produces low amount of ethanol compared with the present study**.** have reported the production of 20.0±0.2 g $L^{-1}$ of ethanol by *S. cerevisiae* after 6 hours of fermentation on the alkali treated cellulose fraction of cashew apple bagasse. Respiratory-deficient mutant strains of wine yeast *S. cerevisiae* produce ethanol yields of 18.8 ± 0.8% in 3.5 weeks period. Immobilized yeast cells of *S. cerevisiae* produce 7.62 % yield of ethanol under optimized fermentation conditions utilizing cashew apple juice as the substrate. The Jerusalem artichoke stalks after acidic hydrolysis and fermentation using *S. cerevisiae* produce 35 g/L of ethanol within 8–9 h. Surface-engineered yeast *S. cerevisiae* when used with uncooked raw starch yields 53 g/L of ethanol in 7 days. When Kinnow waste and banana peels are used by simultaneous saccharification and fermentation using cellulase and co-culture of *Saccharomyces cerevisiae* and *Pachysolen tannophilus*, the ethanol production is registered at 26.84 g.$L^{-1}$ within 48 hours (Sharma et al. 2007).

The commercial production of bioethanol from lignocellulosic hydrolysates by yeast requires the strains that can ferment both hexose and pentose sugars in the hydrolysate, with a high ethanol yield and specific ethanol productivity (Hahn-Hägerdal et al. 2007). Yeasts are potential candidate that are used in fermentation and can be used for ethanol production. For example, *Saccharomyces cerevisiae* efficiently converts both glucose and mannose into ethanol, but is unable to convert xylose into ethanol, hence it is not considered as an ideal candidate for ethanol production. The ability of the fermenting microorganisms to utilize the xylose available from the hydrolysate is a vital factor for increasing the economically competitive production of cellulosic ethanol. However, some of the yeast species, e.g. *Pichia stipitis* and *Candida shehatae*, are capable of converting xylose into ethanol. Among these, *P. stipitis* is able to ferment glucose, xylose, mannose, galactose and cellobiose, and also has the ability to produce cell mass from L-arabinose, but not ethanol (Agbogbo and Coward-Kelly 2008). The results of the present study revealed that the alcohol production varied significantly between the species ($P<0.05$). This species variation observed in the present study may be attributed to various sugar utilization pattern and to the presence of the genes encoding

xylanase, endo-1,4-β-glucanase, exo-1,3-β-glucosidase, β-mannosidase, and α-glucosidase as affirmed in the case of *P. stipitis*. In the present study we found that another species of the genus *Pichia, P. salcaria* is the efficient strain for ethanol production, which may be due to genetic similarity.

The sawdust is mainly composed of lignocellulose which consists of lignin, hemicellulose and cellulose (Betts *et al.* 1991; Sun and Cheng 2002). Of the three components, lignin is the most recalcitrant to degradation whereas cellulose, because of its highly ordered crystalline structure, is more resistant to hydrolysis than hemicellulose. Alkaline (Chahal 1992) and acid (Nguyen 1993; Grethlein and Converse 1991) hydrolysis methods have been used to degrade lignocellulose. Due to the close association of cellulose and hemicellulose with lignin in the plant cell wall, pretreatment is necessary to make these carbohydrates available for enzymatic hydrolysis and fermentation. Hence, pretreatment is an important step in the bioconversions of lignocellulosic materials to ethanol or any other chemical products (Grethlein and Converse 1991; Grethlein 1984; Smith *et al.* 1987). Among various types of processing of the sawdust used for the alcohol production, sawdust processed with 0.8% diluted phosphoric acid showed the maximum production of alcohol at all the concentrations of sawdust tested at varied time intervals (Figs. 1 and 2). In support of this, Arthe *et al.* (2008) have found that 0.8% dilute acid pretreatment has a greater influence than concentrated acid on the sugar release through enzymatic hydrolysis of the cotton waste. When 0.8% dilute acid is mixed with sawdust, it hydrolyses hemicellulose to xylose and other sugars. Thus hemicellulosic fraction of plant cell wall is depolymerised that results in the enhancement of cellulose digestibility in the residues (Nigam 2002; Sun and Cheng 2002; Saha *et al.* 2005).

Yeasts are potential candidate for ethanol production by utilizing cellulose substrate. In the present study, sawdust was supplied as cellulose substrate and the marine yeast, *Pichia salcaria* produced ethanol at various concentrations of sawdust. However the maximum production was obtained when 2% of sawdust was supplied. The utilization of cellulose by microorganisms involves a substantial set of fundamental phenomena beyond those associated with enzymatic hydrolysis of cellulose (Lynd *et al.* 2002). Cellulase is the enzyme involved in hydrolysis of cellulose. It is a mixture of cellulolytic enzymes whose synergistic action is required for effective breakdown of substrate to its monomeric units. In order to get better fermentation performance from this substrate, varied hydrolysis time is required for the performance of cellulolytic enzymes. In the present study, incubation period of 72h showed maximum amount of ethanol production coinciding with cellulase activity (Table 2). Thus cellulase seemed to play a major role in ethanol production when sawdust was used as substrate. This view was substantiated with results from SDS-PAGE analysis which showed a prominent band of 68 kDa after 72h of fermentation, showing similarity with the molecular weight of the enzyme cellulase. However, after 24 and 48h of fermentation, two prominant bands were present with molecular weights of 68, and 150 kDa and after 48h one new band with molecular weight of 54kDa was clearly seen and these protein are similar to the molecular weights of the enzymes cellulase, invertase and zymase respectively (Fig.2). Since sawdust is composed of lignin, hemicellulose and cellulose, it requires multiple enzymes for its effective action. It has already been reported that, the yeast contains invertase enzyme, which acts as a catalyst and helps to convert the sucrose sugars into glucose and fructose (Yanase *et al.* 1995). Similarly, have purified extracellular invertase from yeast *Zymomonas mobilis*. cell-free extracts realized that inorganic phosphate was necessary for glycolysis and that fermentation requires the presence of both a heat-labile component they called "zymase" and a low molecular weight, heat-stable fraction called "cozymase." (It was later shown that zymase contains a number of enzymes whereas cozymase consists of metal ions, ATP, ADP, and coenzymes such as NAD.) Otto Fritz Meyerhof (1884-1951). This produces fructose and glucose sugars and subsequently ethanol and carbon dioxide in the yeast (Cheng *et al.* 2007). However, after 72h of fermentation, only a single prominent band with a molecular weight of 68kDa was produced which is similar to that of cellulase. After 96 and 120h of fermentation, fragmented bands were present, in which none of them were prominent, and this revealed that enzyme activity was almost tampered after 72 hours. The present study indicated that apart from cellulases, xylanase and invertase were also involved in ethanol production. Thus an enzymatic cocktail plays a vital role in ethanol production and this requires further detailed research and detailed mechanism of the enzymatic hydrolysis to bring out the possible utility of marine yeast as an economically viable and ecologically sustainable option in the bioethanol production.

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