

Original Research Article

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## Screening and Identification of Microorganisms for Enhanced Bioethanol Production from Pond Reeds

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### ABSTRACT

#### Keywords

*Phragmites australis*, Lignino cellulose, Fermentation, Bioethanol, Phylogenetic tree.

#### Article Info

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*Phragmites australis*, common pond reed with high lignocelluloses content is suitable for bioethanol production. In the present study pretreated pond reed was first fermented to sugars using cellulose and xylan degrading microorganisms isolated from cow dung and organic kitchen waste. Further fermentation of sugars to ethanol was performed using combinations of *Sachharomyces cerevisiae*, *Trichoderma reesei* and *Pichia stipitis*. The isolated culture, O<sub>2.1</sub> along with the combination of *Trichoderma ressei* and *Pichia Stipitis* exhibited maximum yield of ethanol (3.53gm %). Further identification of O<sub>2.1</sub> was done by 16S-rDNA sequencing. The deduced sequence was used to retrieve the homologous sequences and to construct a phylogenetic tree. The results suggest the use of O<sub>2.1</sub> for better ethanol production from pond reeds which can also help in value addition to the waste.

### Introduction

Rapid industrialization and the limited availability of the natural energy resources has increased the worldwide demand for an alternative energy source. Bioethanol is an important renewable fuel easily obtained from lignocellulosic biomass wastes at relatively low cost and can partly replace fossil derived fuels. (Ashish *et al.*, 2009)

From an economic perspective, wetland perennial grass like pond reeds seems to be the interesting source of biomass (Bothast R.J., 1998). *Phragmites australis* commonly called pond reed is a tall, thin, perennial grass found in wetlands. Due to its worldwide dominance, high lignocellulose content and high productive biomass it can be

a cheap and readily available raw material for biofuel production. Reed has 31.5 % hemicellulose and 49.4 % cellulose and rest is lignin (Tutt and Olt., 2011). Reed has been used for centuries as a fodder in summer, the stems have traditionally been harvested in winter as a raw material for crafts and for construction materials including roofing. Moreover, *Phragmites australis*, out competes the native vegetation and lowers the local plant biodiversity (Köbbing *et al.*, 2013). It is also a source of renewable energy that is unlikely to compete with food production.

*Phragmites australis* form dense thickets of vegetation allowing areas to turn into reed

monoculture very quickly blocking light to other plants making it unsuitable habitat for native fauna which can be easily avoided if common reed is used for ethanol production. Reed biomass can be used as an energy source in three ways, namely by combustion, biogas production and biofuel production.

All stems and leaves can be used regardless of length or diameter. Reed is so-called “second generation” biofuel as it is produced from non-food biomass (e.g. reed) or agricultural residues, in contrast to the “first generation” fuels, which were derived from food crops (e.g. maize).

Glucose for biofuel production can be obtained from reed cellulose by wet oxidation followed by enzymatic hydrolysis saccharification and fermentation (Costa-Ferreira *et al.*, 2007, Szijártó *et al.*, 2009).

Thus, Reed is potentially important in the renewable resources market unlikely to compete for land that is useful for food production (Ganguly A, 2012)

The goal of this study is to find an alternative source of fuel from lignocellulosic biomass by a low cost technique which will be feasible in the developing countries and moreover can help in value addition to the waste. Further the study aims to isolate and identify bacterial strain that help in the saccharification of the reed substrate thereby enhancing ethanol production.

## **Materials and Methods**

### **Microbial cultures and plant material**

*Sachharomyces cerevisiae* NCIM 3044, *Trichoderma reesei* NCIM 1052 and *Pichia stipitis* NCIM 3497 cultures were purchased from NCIM, Pune, India. *Phragmites australis* (Figure 1) were collected from Cipla

Lake, Bangalore, shade dried, pulverized and stored in airtight container for further use.

### **Isolation of cellulose and xylan degrading bacteria from organic waste and cow dung**

Organic kitchen waste was collected from the composting ground inside Garden City University Campus, Bangalore and cow dung was collected from cow shed near Bhattarahalli, Bangalore.

In set A, cellulose degrading bacteria was isolated and in set B, xylan degrading bacteria was isolated. 1gm each of cow dung and organic wastes were inoculated separately into the 20 ml basal salt medium containing cellulose powder and xylan respectively as sole source of carbon for enrichment of cellulolytic and xylanolytic bacteria. The plates were incubated for 7 days in the incubator at 37°C. (Vaishali Randive, *et al.*, 2015). A loopful sample of the bacteria from respective flasks were streaked on CMC agar and Xylan agar separately.

### **Qualitative screening of cellulolytic and xylanolytic bacteria**

Isolates with highest cellulolytic and xylanolytic activity were screened qualitatively by well plate method.

### **Screening by well plate method**

10µl each of individual cellulolytic isolate and xylanolytic isolate was poured into the bored wells of CMC agar plate and Xylan agar plate respectively. The plates were incubated at 37°C for 24- 48 hrs and were flooded with Gram's iodine.

The zone of clearance around colonies were measured. Isolates which showed highest zone of clearance on respective plates were selected for saccharification of pond reed

substrate. (Faridha Begum *et al.*, 2013; Gupta *et al.*, 2011)

### **Pre-treatment of substrate (pond reed)**

Pretreatment was performed following the method of *Umamaheswari.M*, Pond Reeds were collected, washed, shade dried, pulverized and stored in airtight container at room temperature for further assay.

20gm reed sample was added to 330 ml of dilute sulfuric acid (4%) in twelve 1000 ml flask and autoclaved at 121°C for 20mins.

All the 12 flasks were kept at room temperature for 15hrs. Each of the hydrolysate was filtered, neutralized with 1N NaOH, refiltered and washed with sterilised water. The neutralised pre-treated reed cakes were separately dissolved in 200ml of autoclaved basal salt media.

### **Saccharification**

Saccharification was performed following the method of *Syed Shah Minallah Alvil*. Four sets of flasks were set for the process each with different combination of isolated strains and reeds. One set of 3 flasks containing pretreated reed and 20ml of LB were pre inoculated with the highest cellulolytic activity isolates from organic waste. Another set of 3 flasks containing pretreated reed were added with 20ml of LB pre inoculated with the highest cellulolytic activity isolates from cowdung.

Next set of 3 flasks containing pretreated reed were added with 20ml of LB pre inoculated with the highest xylanolytic activity isolates from organic waste.

Last set of 3 flasks containing pretreated reed were added with 20ml of LB pre inoculated with the second highest xylanolytic activity showing isolate from organic waste. All the

12 flasks were kept at 37°C for 4days for saccharification.

### **Quantitative estimation of total sugars and xylose**

After saccharification was completed, all the flasks were tested for the presence of sugar.

Total sugar produced in the flasks inoculated with isolated strains of both cellulolytic and xylanolytic bacteria were determined by Anthrone method using glucose as standard(*Gupta et al.*, 2011); xylose produced in the flasks inoculated with isolated strains of xylanolytic bacteria were determined by Phloroglucinol method(*Eberts et al.*, 1979) using xylose as standard.

### **Production of ethanol by fermentation process**

Hydrolysed reeds were fermented with 12 different combinations of 4 bacterial isolates from cow dung and organic waste ( $O_{2.1}$ ,  $CD_{50}$ ,  $O_b$ ,  $O_c$ ) and 3 fungal cultures (*Sachharomyces cerevisiae* (S.C), *Trichoderma reesei* (T.C) and *Pichia stipitis*(P.C). They are as follows : Flask A1, A2, A3, A4, A5, A6, B1, B2, B3, B4, B5, B6 has  $O_{2.1}$  + *Saccharomyces cerevisiae*(S.C),  $O_{2.1}$  + *Trichoderma reesei*(T.R),  $O_{2.1}$  + *Pichia stipitis*(P.C) + *Trichoderma reesei* (T.R),  $CD_{50}$  + *Saccharomyces cerevisiae*,  $CD_{50}$  + *Trichoderma reesei*,  $CD_{50}$  + *Pichia stipitis* (P.C) + *Trichoderma reesei*(T.R),  $O_{2.1}$  +  $O_b$  + *Saccharomyces cerevisiae*(S.C),  $O_{2.1}$  +  $O_b$  + *Trichoderma reesei*(T.R),  $O_{2.1}$  +  $O_b$  + *Pichia stipitis*(P.C) + *Trichoderma reesei*(T.R),  $O_{2.1}$  +  $O_c$  + *Saccharomyces cerevisiae*,  $O_{2.1}$  +  $O_c$  + *Trichoderma reesei*,  $O_{2.1}$  +  $O_c$  + *Pichia stipitis* (P.C) + *Trichoderma reesei*(T.R) respectively.

These flasks were incubated at 27°C for 8 days (*Mervate A*, 2014). After fermentation, distillation was done and total alcohol percentage was calculated with specific

gravity method. Ethanol concentration produced in each set after fermentation were determined by dichromate assay (Bennett, 1971; Pilone, 1985).

### Identification of bacterial isolate with highest cellulytic and xylanolytic activity

The strain showing highest cellulytic, xylanolytic activity and gave better yield of ethanol was selected for species identification. Genomic DNA of the bacterium was isolated and its 16S-rDNA was amplified (Mamata Pasayat, et al., 2016).

The PCR products were sequenced from Stellixir Biotech Pvt. Ltd., Bangalore, India. The deduced sequence was subjected to BLAST algorithm from the National Centre of Biotechnology Information, (<http://www.ncbi.nlm.nih.gov>) to retrieve for homologous sequences and also subjected to construct a phylogenetic tree by the help of Clustal Omega bioinformatics tool (Faridha Begum et al., 2013).

## Results and Discussion

### Isolation and screening of cellulose and xylan degrading bacteria

4 isolated species from organic waste (O<sub>1</sub>, O<sub>2.1</sub>, O<sub>9</sub>, O<sub>50</sub>) and 3 isolated species from cow dung sample (C<sub>1</sub>, C<sub>2</sub>, CD<sub>50</sub>) were screened for cellulose degrading activity. Among the isolates from organic waste O<sub>2.1</sub> showed maximum zone of clearance (4.5cm) followed by O<sub>1</sub> (4.2cm) (Figure 2A). Out of the 3 isolates from cow dung sample, CD<sub>50</sub> showed maximum zone of clearance (2.1cm) followed by C<sub>1</sub> (1cm diameter) (Figure 2B).

Further to the study, Xylan degrading organisms were isolated from cow dung and organic waste. Out of the 3 isolated species from organic waste (O<sub>b</sub>, O<sub>c</sub>, O<sub>a</sub>), O<sub>b</sub> showed significant zone of inhibition (1.3cm

diameter) followed by O<sub>c</sub> (0.4cm diameter) (Figure 3).

No species was isolated from cow dung that can degrade xylan.

O<sub>2.1</sub> and CD<sub>50</sub> were checked for growth on xylan agar plates. Only O<sub>2.1</sub> could grow indicating O<sub>2.1</sub> has ability to degrade xylan as well as cellulose.

### Estimation of total sugars and xylose

Total sugars from the saccharified reeds were estimated by Anthrone method. From the calibration curve, the concentration of total sugar produced by the pretreated reed inoculated with O<sub>2.1</sub>, CD<sub>50</sub>, (O<sub>2.1</sub>+O<sub>b</sub>), (O<sub>2.1</sub>+O<sub>c</sub>) were calculated and mentioned in (Table 1 and Figure 4). After pretreatment of reed with dil. H<sub>2</sub>SO<sub>4</sub> (4%) bacterial species namely O<sub>2.1</sub>, CD<sub>50</sub>, O<sub>2.1</sub>+O<sub>b</sub> and O<sub>2.1</sub>+O<sub>c</sub> were inoculated into the flasks for the hydrolysis and the amount of xylose formed were estimated by phluroglucinol method. From the calibration curve, the concentration of xylose produced by the pretreated reed inoculated with O<sub>2.1</sub>, CD<sub>50</sub>, (O<sub>2.1</sub>+O<sub>b</sub>), (O<sub>2.1</sub>+O<sub>c</sub>) are calculated and mentioned in (Table 2 and Figure 5)

### Estimation of total percentage of alcohol and ethanol content

When total percentage of alcohol was estimated by specific gravity method, the combination of *Trichoderma ressei*, *Pichia Stipitis*, the bacterial isolate O<sub>2.1</sub> and O<sub>b</sub> showed maximum content of 12.46 gm% followed by the combination of O<sub>2.1</sub>, O<sub>b</sub> and *Trichoderma ressei* yielding 11.13gm% (Figure- 6). High ethanol yield was observed for the combination of *Trichoderma ressei*, *Pichia Stipitis* and the bacterial isolate O<sub>2.1</sub> showed maximum of 3.53gm % followed by the combination of O<sub>2.1</sub>, O<sub>b</sub>, *Trichoderma ressei* and *Pichia Stipitis* with 3.48gm% of ethanol (Table 3 and 4, Figure 7A and 7B).

**Table.1** Total sugar produced after saccharification

| S.No | Inoculum Used For Saccharification  | Concentration of total sugar( $\mu\text{g/ml}$ ) |
|------|-------------------------------------|--|
| 1.   | O <sub>2.1</sub>                    | 184.68   |
| 2.   | CD <sub>50</sub>                    | 164.82   |
| 3.   | (O <sub>2.1</sub> +O <sub>b</sub> ) | 427.2  |
| 4.   | (O <sub>2.1</sub> +O <sub>c</sub> ) | 625.7  |

**Table.2** Xylose concentration after saccharification

| S.No | Inoculum used for Saccharification  | Concentration of xylose sugar( $\mu\text{g/ml}$ ) |
|------|-------------------------------------|---|
| 1.   | O <sub>2.1</sub>                    | 84.62   |
| 2.   | CD <sub>50</sub>                    | Nil   |
| 3.   | (O <sub>2.1</sub> +O <sub>b</sub> ) | 223.01  |
| 4.   | (O <sub>2.1</sub> +O <sub>c</sub> ) | 143.93  |

**Table.3** Yield of Gm% of Ethanol in flask A1-A6

| Sl.No | Saccharification and fermentation                                 | OD at 590nm | Gm % of ethanol |
|-------|---|-------------|-----------------|
| A1    | O <sub>2.1</sub> + <i>Saccharomyces cerevisiae</i>                | 0.19        | 2.24            |
| A2    | O <sub>2.1</sub> + <i>Trichoderma ressei</i>                      | 0.26        | 3.28            |
| A3    | O <sub>2.1</sub> + <i>Trichoderma ressei</i> + <i>P.stipitis</i>  | 0.28        | 3.53            |
| A4    | CD <sub>50</sub> + <i>Saccharomyces cerevisiae</i>                | 0.10        | 1.21            |
| A5    | CD <sub>50</sub> + <i>Trichoderma ressei</i>                      | 0.12        | 1.47            |
| A6    | CD <sub>50</sub> + <i>Trichoderma ressei</i> + <i>p. stipitis</i> | 0.18        | 2.22            |

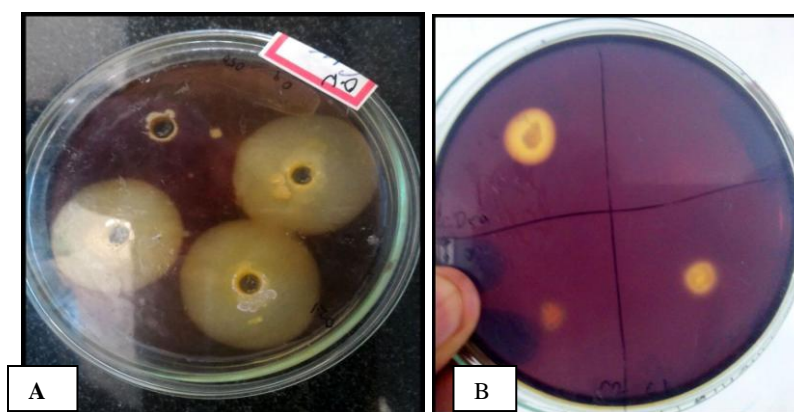
**Table.4** Gm% of Ethanol yield in flask B1-B6

| Sl no. | Inoculum used for saccharification and fermentation respectively                  | OD at 590nm | Gm % of ethanol |
|--------|---|-------------|-----------------|
| B1     | O <sub>2.1</sub> +O <sub>b</sub> + <i>Saccharomyces cerevisiae</i>                | 0.08        | 1.13            |
| B2     | O <sub>2.1</sub> +O <sub>b</sub> + <i>Trichoderma ressei</i>                      | 0.24        | 2.98            |
| B3     | O <sub>2.1</sub> +O <sub>b</sub> + <i>Trichoderma ressei</i> + <i>P. stipitis</i> | 0.27        | 3.48            |
| B4     | O <sub>2.1</sub> +O <sub>c</sub> + <i>Saccharomyces cerevisiae</i>                | 0.20        | 2.47            |
| B5     | O <sub>2.1</sub> +O <sub>c</sub> + <i>Trichoderma ressei</i>                      | 0.14        | 1.85            |
| B6     | O <sub>2.1</sub> +O <sub>c</sub> + <i>Trichoderma ressei</i> + <i>P. stipitis</i> | 0.17        | 2.13            |

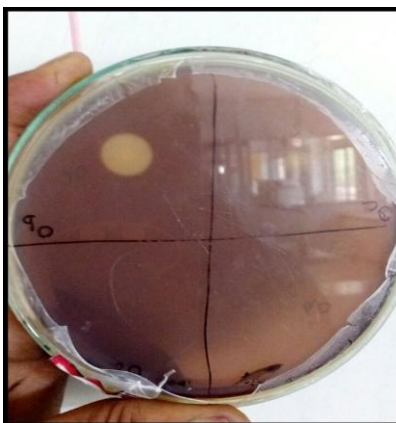
**Fig.1** *Phragmites australis* (Pond reeds)



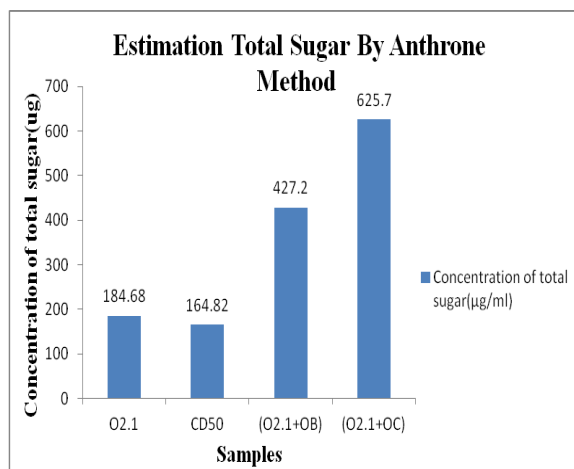
**Fig.2** Qualitative screening of cellulose degrading bacterial isolates A) O<sub>2.1</sub> isolate showing maximum cellulose degradation B) CD<sub>50</sub> isolate showing maximum cellulose degradation



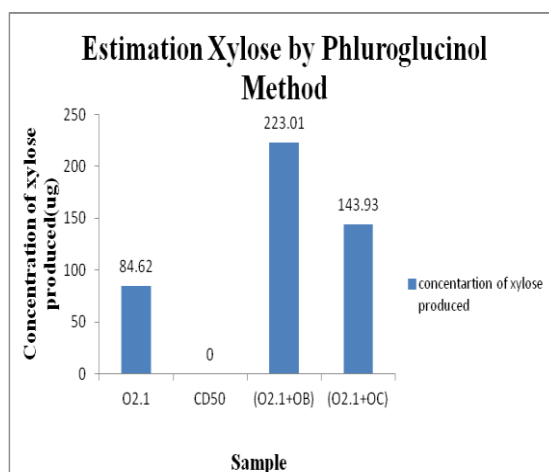
**Fig.3** Qualitative screening of xylan degrading bacterial isolates (O<sub>b</sub> isolate showing maximum Xylose degradation)



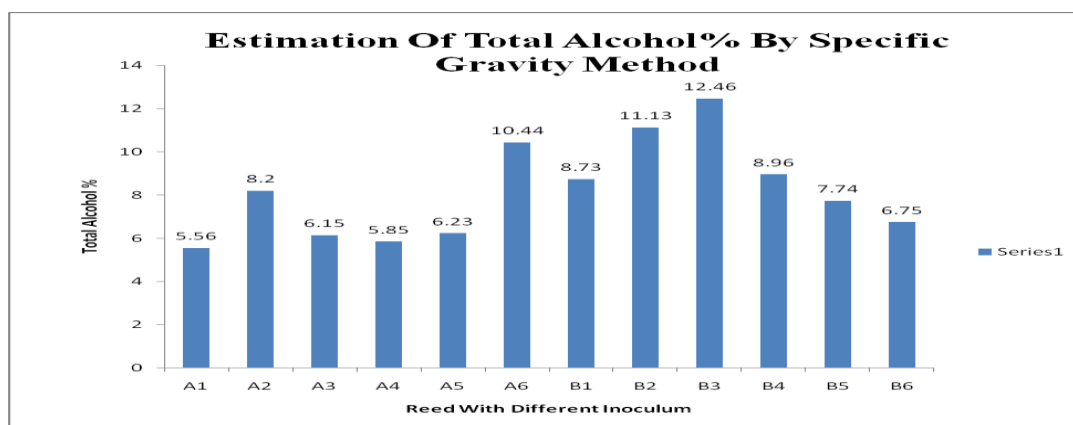
**Fig.4** Total sugar produced by saccharification of pond reed using various isolates  
From organic waste and cow dung



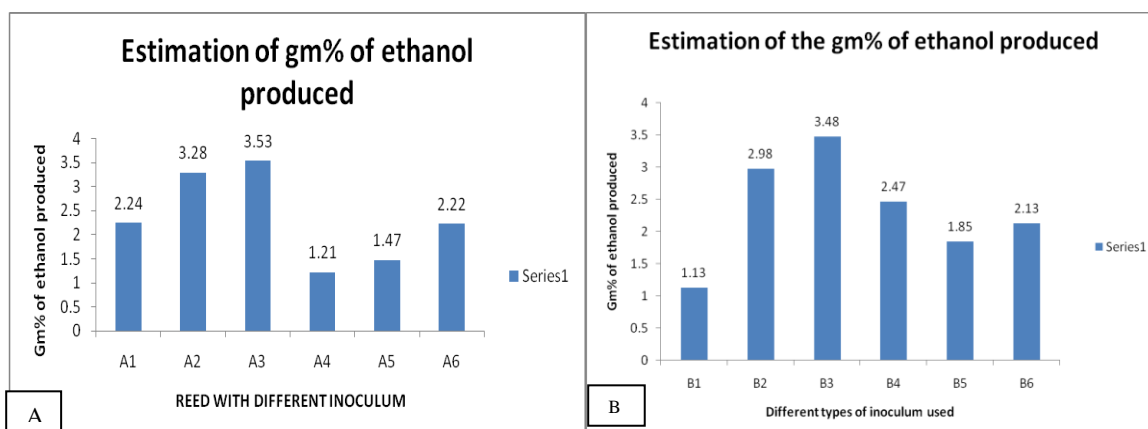
**Fig.5** Xylose produced by saccharification of pond reed using various isolates  
From organic waste and cow dung



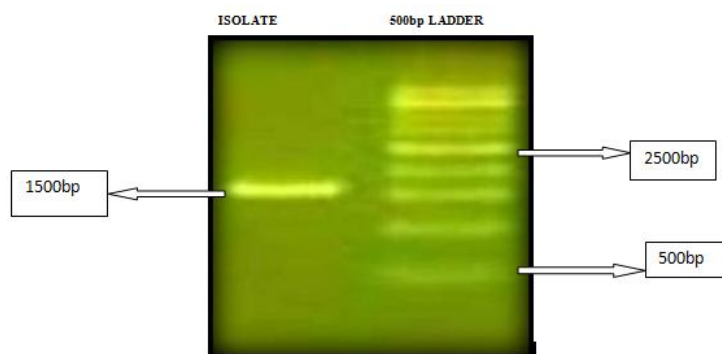
**Fig.6** Total alcohol in flask A1-A6 and flask B1-B6 measured by specific gravity method



**Fig.7** A) Ethanol yield in flask A1-A6. B) Ethanol yield in flask B1-B6 measured by dichromate method



**Fig.8** 16s-rDNA amplication product of isolate O<sub>2.1</sub>



**Fig: 16s-rDNA amplication product**

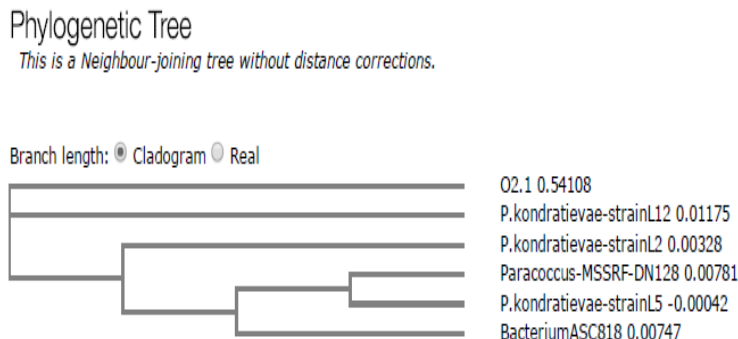
**Fig.9** NCBI BLAST analysis of 16S rRNA gene of isolate O<sub>2.1</sub>

### Descriptions

Sequences producing significant alignments:

| Description   | Max score | Total score | Query cover | E value | Ident | Accession                  |
|---|-----------|-------------|-------------|---------|-------|----------------------------|
| Paracoccus sp. MSSRF DN128 16S ribosomal RNA gene, partial sequence         | 1000      | 1000        | 77%         | 0.0     | 91%   | <a href="#">KU131291.1</a> |
| Paracoccus kondratievae strain L12 16S ribosomal RNA gene, partial sequence | 1000      | 1000        | 77%         | 0.0     | 91%   | <a href="#">JX646644.1</a> |
| Paracoccus kondratievae strain L5 16S ribosomal RNA gene, partial sequence  | 1000      | 1000        | 77%         | 0.0     | 91%   | <a href="#">JX646642.1</a> |
| Paracoccus kondratievae strain L2 16S ribosomal RNA gene, partial sequence  | 1000      | 1000        | 77%         | 0.0     | 91%   | <a href="#">JX646641.1</a> |
| Bacterium ASC818 16S ribosomal RNA gene, partial sequence                   | 1000      | 1000        | 77%         | 0.0     | 91%   | <a href="#">JQ796003.1</a> |

**Fig.10** Phylogenetic tree construction for the isolate O<sub>2.1</sub>



### Molecular identification of the bacterial isolate O<sub>2.1</sub>

The Genomic DNA was obtained by using Chromous Genomic DNA isolation kit. The amplified PCR products of the bacterial isolate was analyzed by electrophoresis and photographed under UV transilluminator. An Amplified DNA band of 1.5 kb was observed (Figure 8).

16S rDNA sequencing of PCR Amplicon was done at Stellixir Biotech Pvt. Ltd., Bangalore, India. BLAST analysis of 16S rDNA of O<sub>2.1</sub> isolate showed 91% sequence similarity with four species of *Paracoccus* and *Bacterium* ASC818 with a query coverage of 77 % (Figure 9).

Further with the phylogenetic tree analysis the isolate showed *P.kondratievae* as the nearest neighbor as shown in figure 10.

From an economic perspective, wetland perennial grass like pond reeds *Phragmites australis*, seems to be the interesting source of biomass (Bothast R.J., 1998). *Phragmites australis* was saccharified and fermented to yield alcohol using the bacterial isolates from organic waste and cow dung.

Out of the 4 isolates as O<sub>2.1</sub> showed maximum zone of clearance on CMC agar plate.

O<sub>2.1</sub> could grow on xylan agar plate as none of the isolates from cow dung could do indicating that O<sub>2.1</sub> has the ability to degrade xylan as well as cellulose. This would explain for the maximum yield of total sugars and Xylose produced with the hydrolysis of reed. Mixed culture of O<sub>2.1</sub> and O<sub>b</sub> also enhanced the production of total sugars and xylose than any other culture in combination, evidencing the Saccharification efficiency of O<sub>2.1</sub>.

Thus it could be concluded that O<sub>2.1</sub> culture when used along with the combination of *Trichoderma reesei* and *Pichia Stipitis*, maximum yield of ethanol was obtained. Isolated genomic DNA from O<sub>2.1</sub> was amplified and a 1.5 kb of amplified product was obtained. Homology search was done by BLAST analysis, which showed that 16S ribosomal RNA gene partial sequence of O<sub>2.1</sub> species showed 91% similarity with 16S ribosomal RNA gene partial sequence of five other species in NCBI database with a query coverage of 77%.

Therefore, this combination can be used for better ethanol production from pond reeds. In this process even the waste reed around the ponds and lakes could be used up efficiently. Thus this technique is effective in reducing the population of common reed and in value addition to the waste, using low cost methods, which will be feasible in the developing

countries thus giving an alternative source of bioethanol in near future.

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