



# A novel marine bacterium *Isoptericola* sp. JS-C42 with the ability to saccharifying the plant biomasses for the aid in cellulosic ethanol production



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## ABSTRACT

The ever growing demands for food products such as starch and sugar produces; there is a need to find the sources for saccharification for cellulosic bioethanol production. This study provides the first evidence of the lignocellulolytic and saccharifying ability of a marine bacterium namely *Isoptericola* sp. JS-C42, a Gram positive actinobacterium with the cocci cells embedded on mycelia isolated from the Arabian Sea, India. It exhibited highest filter paper unit effect, endoglucanase, exoglucanase, cellobiohydrolase,  $\beta$ -glucosidase, xylanase and ligninase effect. The hydrolytic potential of the enzymes displayed the efficient saccharification capability of steam pretreated biomass. It was also found to degrade the paddy, sorghum, *Acacia mangium* and *Ficus religiosa* into simple reducing sugars by its efficient lignocellulose enzyme complex with limited consumption of sugars. Production of ethanol was also achieved with the *Saccharomyces cerevisiae*. Overall, it offers a great potential for the cellulosic ethanol production in an economically reliable and eco-friendly point-of-care.

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## 1. Introduction

Cellulose is a structural framework of plant cell wall comprising of 35–50% weight basis of plant material [1] and one of the major constituents of renewable biomass. The major contribution for structural component in the cell wall is a cellulose complex comprising of linear polymer of  $\beta$  (1→4) glucose units. In plant cell walls, the cellulose contributes a microcrystalline structure and its component cellulose 1 $\alpha$ , one of the stable isoform, which aids to 70% crystalline thus makes them hard material for saccharification [2]. The microcrystalline structure of cellulose is more difficult to hydrolyze economically into reducing sugars when compared to starch [3].

Generally cellulose hydrolytic enzymes are produced naturally by a wide range of microbial communities, including bacterial and fungal species. They are known to biosynthesize different types of cellulase enzymes, which have distinct metabolic actions on the breakdown of cellulose [4,5] and these enzymes play a key role in the large scale conversion of plant biomass into simple, reducing sugars and facilitate the possible opportunity in modern

tools of biotechnological applications to meet the growing fuel demands [6]. Due to the high cost, ever growing demand and depletion of fossil fuel resources with global warming problems by the increased emission of greenhouse gases (GHG); the spread of cost-effective technologies for producing alternate renewable fuels such as ethanol from cellulosic biomass feedstocks have emerged both at research and industrial scale.

The second-generation biofuel, cellulosic ethanol is produced from non food based, renewable, fibrous lignocellulosic plant biomass. The lignocellulosic plant biomass is not used as a human food and is available as vast quantity at cheaper cost; hence there is no significant negative impact on the global food security [7]. Ethanol production from lignocellulosic materials involved a multistep process in which the size of the biomass should be reduced by pretreatment, enzymatic hydrolysis of cellulose and hemicellulose to simple sugars and finally conversion of released sugars into ethanol.

The crop stubbles left out in the field after harvesting (rice and sorghum) and tree residues were routinely burned around the globe which creates serious pollution and health issues [8]. However, all these crop residues represent a biofuel feedstock for ethanol production due to their cheaper cost, easy availability, easy processability, non-hazardous, non-abrasive, recyclable and environmental friendly nature. The aim of the present study is to

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evaluate the enzymatic saccharification of steam pretreated cellulosic plant biomass by the newly isolated halotolerant marine actinomycetes bacterium, *Isoptericola* sp. JS-C42 and simultaneous conversion into ethanol by fermentation assisted by *Saccharomyces cerevisiae*.

## 2. Materials and methods

### 2.1. Sample collection and organism isolation

Marine sediment samples were collected randomly from the Arabian Sea at Kanyakumari, India. The samples were transferred to the laboratory under sterile conditions and stored at 4 °C until use. Soil samples were serially diluted in sterile distilled water and spread plated over the slightly modified medium containing 10.0 g cellulose, 2.0 g NaNO<sub>3</sub>, 0.5 g KCl, 1.0 g K<sub>2</sub>HPO<sub>4</sub>, 0.5 g MgSO<sub>4</sub>, 20 μM FeSO<sub>4</sub>, 15 g agar, 1 L seawater, pH 7.6 and 15 μg nalidixic acid to prevent the growth of Gram-negative bacteria [9] and incubated at 28 ± 2 °C for 3–5 days.

### 2.2. Cellulolytic assay by Gram's iodine test

Cellulase activity was screened qualitatively by growing the isolate on the cellulose agar (10.0 g cellulose, 2.0 g NaNO<sub>3</sub>, 0.5 g KCl, 1.0 g K<sub>2</sub>HPO<sub>4</sub>, 0.5 g MgSO<sub>4</sub>, 20 μM FeSO<sub>4</sub>·5H<sub>2</sub>O, 15 g agar per L, pH 7.6) at 30 °C for 48 h. After incubation, the cellulose agar plates were flooded with 3.0 mL Gram's iodine solution (KI 2.0 g, iodine 1.0 g in 300 mL distilled water) for 5 min [10] and the appearance of clearance zone was observed. The experiment was done in triplicates.

### 2.3. Glucanohydrolase activity assay by Congo red

The carboxymethyl cellulose (CMC) agar (10.0 g CMC, 2.0 g NaNO<sub>3</sub>, 0.5 g KCl, 1.0 g K<sub>2</sub>HPO<sub>4</sub>, 0.5 g MgSO<sub>4</sub>, 20 μM FeSO<sub>4</sub>, 15.0 g agar, 1.0 L water, pH 7.6) was used for the evaluation of cellulase production by inoculating the bacterial isolate. The CMC-agar plates were incubated at 28 ± 2 °C for 3 days. The clearance zone displaying cellulase activity was detected by staining the CMC-agar plate with 0.1% Congo red solution for 15 min and destained with 1 M NaCl for 20 min [11]. The extracellular enzyme produced by the cellulolytic strain JS-C42 in modified cellulose medium was concentrated by 60% ammonium sulphate precipitation and dialyzed using a dialysis tubing with a molecular weight cutoff (MWCO) of 12,000 (globular proteins) (Sigma-Aldrich, USA) bathed in a citrate buffer. The endo-β-1,4-glucanase activity of the concentrated enzyme solution was determined by the agar plug well assay using 50 μg/10 μL enzyme solution to the agar plug wells supplemented with 1.0% CMC.

### 2.4. Phylogenetic characterization by 16S rRNA gene typing

Genomic DNA of the isolate was extracted by the modified protocol described by Sharma and Singh [12] and the modified step in the protocol was the resuspension of cell pellets in 100 μL sucrose TE buffer (25 mM Tris, pH 8.0; 10 mM EDTA, pH 8.0 and 300 mM sucrose) containing 2.0 mg mL<sup>-1</sup> lysozyme and the suspension was incubated at 37 °C for 15 min. The 16S rRNA gene was amplified by PCR as described by Solomon et al. [13] using 16S rRNA gene specific 27F and 1492 universal primers. The amplified product was purified by QIAquick PCR purification kit (Qiagen, USA) according to the manufacturer's instruction, cloned into pGEM-T easy vector (Promega, USA) and confirmed positive clone was sequenced by Applied Biosystems 3730XL DNA analyzer with the sequencing reaction components derived from BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA). Quality of the 16S rRNA gene sequence was analyzed by Pintail

(<http://www.bioinformatics-toolkit.org/Web-Pintail/>). The phylogenetic analysis was performed by BLAST, Ribosomal Database Project-II (RDP-II) database [14] by Neighbor Joining method and Maximum-likelihood analysis was performed with DNAML of PHYLIP 3.68 [15]. The phylogenetic tree was constructed using PHYLIP 3.68 with DNADIST, NEIGHBOR using bootstrapping over 1000 replicates and viewed with the help of Treeview [16]. The 16S rRNA gene sequence of the cellulolytic bacterial isolate JS-C42 was deposited in GenBank under the accession number KC987474.

### 2.5. Assay for enzymatic hydrolysis with the cellulase preparations

The protein extracted from culture filtrate of JS-C42 isolate was concentrated by ammonium sulphate precipitation, desalted by dialysis in 50 mM citrate buffer, pH 4.8 and assayed for the filter paper unit (FPU) activity, exo-β-glucanase, endo-β-1,4-glucanase, cellobiohydrolase, xylanase, β-glucosidase and lignin peroxidase (LiP) using the substrates Whatman No. 1 filter paper (1 cm × 6 cm, 50 mg) strips, Avicel, carboxymethyl cellulose, cellobiose, xylan from beach wood, P-nitrophenyl β-D-glucopyranoside and veratryl alcohol respectively. The protein concentration of the enzyme extract was determined using Quick Start™ Bradford protein assay kit (Bio-Rad, CA, USA) and the enzyme assays were performed in by following the standard methods [17–20].

### 2.6. Pretreatment of plant biomass by steam explosion

The paddy straw, sorghum stubbles, leaves of *A. mangium* and *F. religiosa* were chopped into small pieces, powdered and sieved through 1.0 mm mesh sieves. The ground, sieved plant biomass substrate was pretreated by the steam explosion as described by Sharma et al. [21] by releasing rapid discharge of high-pressure steam to a vessel operated at lower pressure. The exploded biomass substrates were immediately used in the enzymatic saccharification experiment.

### 2.7. Reducing sugar release from plant biomass

The JS-C42 isolate was grown on pretreated paddy straw (1.0%, w/v), paddy straw with glucose (1.0% and 0.03%), leaves of *A. mangium* and *F. religiosa* and microcrystalline cellulose (1% Sigmacell cellulose, Sigma, USA) with the mineral salts 2.0 g NaNO<sub>3</sub>, 0.5 g KCl, 1.0 g K<sub>2</sub>HPO<sub>4</sub>, 0.5 g MgSO<sub>4</sub>, 20 μM FeSO<sub>4</sub> per L, pH 7.6 and incubated at 30 °C, 120 rpm and 1.0 mL culture supernatants were withdrawn once in 6 h, cells were removed by centrifugation at 8000 rpm for 5.0 min and supernatant was subjected to filter sterilization in order to monitor the release of reducing sugars by cellulolytic action of JS-C42 strain.

### 2.8. Cellulosic ethanol conversion

The simple sugars produced by the hydrolytic effect of *Isoptericola* sp. JS-C42 in spent medium at the optimum sugar production stage was transferred to BioFlo® CelliGen® 115 fermentor (New Brunswick, CT, USA) and the fermentation was mediated by *Saccharomyces cerevisiae* MTCC 170 (IMTECH, Chandigarh, India). The seed culture of *S. cerevisiae* MTCC 170 (IMTECH, Chandigarh, India) was prepared in a one-liter Erlenmeyer flask containing 250 mL of YM broth, pH 6.2 ± 0.2 (HiMedia, Mumbai, India), incubated at 30 °C, 150 rpm for 14 h in an orbital shaker incubator (Neolab, India). Then the yeast inoculum was transferred to a BioFlo 115 vessel containing 4.75 L of spent medium of *Isoptericola* sp. JS-C42 containing reducing sugars derived from plant biomass. The fermentor was programmed at 30 °C, aeration rate 2.5 L min<sup>-1</sup> (0.5 vessel vol min<sup>-1</sup>), agitation speed 200 rpm, pH was maintained

at 5.0 using 29% NH<sub>4</sub>OH base solution and the elapsed fermentation time was 72 h. Samples were withdrawn at a particular time interval, filtered through 0.2 μm filters, the alcohol and residual sugar content were analyzed [22]. Ethanol production from steam pretreated biomasses and the relevant energy consumption were analyzed by [23].

### 2.9. Atomic force microscope analysis

For atomic force microscope analysis of bacterial interaction over cellulosic substrate, cover slip was cleaned by sonication, after complete air drying cover slip was treated with piranha solution (3:1 conc. H<sub>2</sub>SO<sub>4</sub> to 30% H<sub>2</sub>O<sub>2</sub> solution) for 15 min, then washed three times with sterile milliQ water and dried in vacuum desiccators. The logarithmic growth phase cultures were pelleted at 5000 rpm for 10 min at 4 °C, washed three times with sterile ultrapure water and diluted up to 10<sup>-3</sup> dilution. To fix the bacterial cells on the desiccated glass cover slip, 10 μl of 10<sup>-3</sup> diluted bacterial culture was gently pipetted and air dried for 12 h. Likewise, 5 μl of the cell suspension was carefully placed on another desiccated cover glass coated with 10 μl sterile tryptic soy broth containing filter sterilized 1% Sigmacell, incubated for 13 h till air dry. Then the samples were observed with preliminary scanning for several times with A100-SGS Atomic Force Microscope (A.P.E Research). Non contact mode images were taken with silicon etched Ultrasharp™ probe tip (MikroMasch, USA) with 10 nm radius and a spring constant of 40 N m<sup>-1</sup> by tapping mode in air at room temperature to measure the height and deflection of the specimen.

## 3. Results and discussion

### 3.1. Sample collection and isolation of cellulolytic microorganism

The bacterial isolates exhibiting cellulolytic activity were isolated from the Arabian Sea, India. Enrichment cultures after 5–10 consecutive transfers exhibited reproducible cellulose hydrolysis as indicated by the extent of the cellulolytic clear zone in agar substrate plates, and time required for cellulose metabolism. We obtained many aerobic cellulolytic microorganisms which were distinguished based on their colony morphology. Among them, a bacterial isolate JS-C42 exhibited highest lignocellulolytic effect. In this study, we are presenting a detailed report of a yellow actinomycete isolate JS-C42. Plating of the cultures of JS-C42 on cellulose agar during subsequent sub culturing also depicted the extensive clearing zones. The clearing zone shown depicted the cellulose solubilization by extracellular enzymes produced by JS-C42 isolate and this result was in accordance with the cellulolytic studies as reported by Sizova et al. [24].

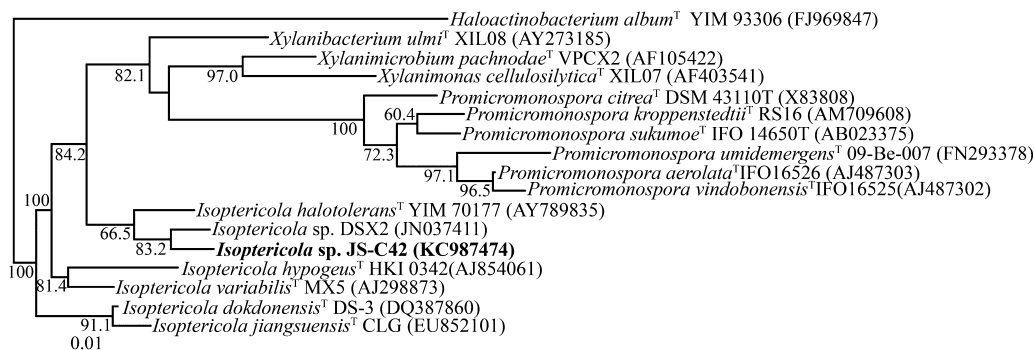
Cellulolytic strain JS-C42 has a smooth surface, pale yellow, circular, opaque colonies and approximately 1.0 mm in diameter after 36 h growth at 28 °C on cellulose supplemented medium. It grew well at pH 7.5–9.0, 28–37 °C and up to 10% NaCl concentration. The cells were Gram-positive, non-motile cocci-shaped, have primary mycelium with no spore and exhibited aerobic growth. The bacterial isolate JS-C42 utilized the starch, casein, urea and lipid molecule such as tributyrin. The utilization of starch, casein, mannitol salt agar, tributyrin, and urea showed that the isolate produced the extra cellular enzymes amylase, protease, lipase and urease to metabolize the polymeric components of the nutrient mixture to monomeric form for the growth.

### 3.2. Sequencing and phylogenetic analysis of 16S rRNA gene of isolate JS-C42

For predicting the phylogenetic position of the isolate JS-C42, the phylogenetic tree (Fig. 1) with its closely related type and non-type strains were analyzed using Ribosomal Database Project. The nucleotide sequence of the 16S rRNA gene of JS-C42 displayed 98.9% sequence identity to the available 16S rRNA gene sequence of the type strain *Isoptericola halotolerans* YIM 70177 and 99.3% sequence similarity to the non type strain *Isoptericola* sp. DSX2. The closely related type strain *Isoptericola halotolerans* YIM 70177 was negative for milk peptonization and starch hydrolysis and its colonies are pale-yellow in color [25]. When compared to the type strain *Isoptericola halotolerans* YIM 70177 and in spite of the 16S rRNA gene sequence identity of 98.9%, the cellulolytic bacterial isolate JS-C42 showed phenotypic differences in cell morphology like intense yellow with distinct mycelium and distinct biochemical properties like positive reaction for milk peptonization and starch hydrolysis. Overall the phylogenetic analysis of cellulolytic bacterial isolate JS-C42 revealed its belongings to the phylum Actinomycetes and denoted as *Isoptericola* sp. JS-C42.

### 3.3. Cellulolytic assay by Gram's iodine test

The cellulose hydrolysis is observed after the 48 h incubation with a zone of the hydrolyzed region of the cellulosic agar medium flooded with Gram's iodine, which produces a bluish-black complex with cellulose but not with hydrolyzed zone containing simple sugars [10]. The clearance zone visualized on 2nd and 3rd day of incubation of bacterial isolate JS-C42 on cellulolytic medium was 2.4 ± 0.4 and 2.5 ± 0.2 cm respectively and high cellulolytic ability exhibited by the bacterial isolate JS-C42 was due to its fast growing ability than the other cellulose degraders.



**Fig. 1.** Phylogenetic tree based on 16S rRNA gene sequences of bacterial isolate JS-C42 and related taxa belonging to the phylum Actinobacteria. The tree was constructed by the neighbor-joining method using Ribosomal Database Project, Phylip and Mega software tools. The numbers represent bootstrap values for each branch, based on data for 1000 trees. Accession numbers for 16S rRNA gene sequences are given for each strain in parentheses. The scale bar shows the number of nucleotide substitutions per site of 1%.

### 3.4. Glucanohydrolase activity assay by Congo red dye

Congo red exhibits a strong interaction with complex polysaccharides composed of contiguous  $\beta$ -(1 $\rightarrow$ 4) linked D-glucopyranosyl entities. It also shows a significant interaction with  $\beta$ -(1 $\rightarrow$ 3), (1 $\rightarrow$ 3)-D-glucan units [11], thus identify the bacterial strains possessing  $\beta$ -(1 $\rightarrow$ 4), (1 $\rightarrow$ 3)-D-glucanohydrolase,  $\beta$ -(1 $\rightarrow$ 4)-D-glucanohydrolase, and  $\beta$ -(1 $\rightarrow$ 3)-D-glucanohydrolase activities. The bacterial isolate JS-C42 showed an efficient cellulolytic zone ( $2.4 \pm 0.2$  cm) by Congo red based assay and it provides a contemporary basis for the assay of endo- $\beta$ -D-glucanase activity exhibited by JS-C42 isolate in an agar medium containing CMC as a substrate. Appearance of yellow clearance zone within 5 min after the addition of enzyme solution in agar plug wells also indicated the isolate JS-C42 displayed the endo- $\beta$ -1,4-glucanase activity.

### 3.5. Lignocellulolytic effect of enzymes produced by the JS-C42

The cellulolytic strain JS-C42 exhibited saccharifying cellulase effect against crystalline cellulose as  $30.71 \mu\text{mol min}^{-1} \text{mL}^{-1}$  ( $\text{IU mL}^{-1}$ ) and it was sixfold higher than the positive control,  $4.95 \mu\text{mol min}^{-1} \text{mL}^{-1}$  ( $\text{IU mL}^{-1}$ ) FPU activity exhibited by *T. reesei*. Recent reports suggest that the minimal amount of 10 FPU is sufficient to convert 1.0 g of cellulosic substrate into glucose at 85% level to produce an efficient ethanol yield [26,27]. The bacterial isolate JS-C42 produced 30.71 FPU activities and this level is above the minimal requirement of FPU for cost effective cellulose bio-transformation into glucose for the ethanol production. Apart from the FPU, the extracellular enzymes produced by the isolate JS-C42 also exhibited endoglucanase, exoglucanase, cellobiohydrolase,  $\beta$ -glucosidase, xylanase and lignin hydrolytic effect (Table 1).

Steam explosion pretreatment was employed mainly to remove the lignin component of the cell wall by opening biomass fibers and improve the release of sugars with less energy utilization. When compared to other pretreatment processes, it offers no chemical usage except water, and avoidance of corrosion causing chemicals such as acids [28]. In this study though the steam pretreatment is not effective in releasing reducing sugars from Acacia, it plays a significant role in other plant biomasses. The steam pretreated lignocellulosic substrates showed the improved saccharification by enzymatic hydrolysis and yielded approximately 70–78% of glucose based on the cellulose content of the pretreated plant biomass. The enzymatic saccharification of pretreated biomass also exhibited 13–33% increased reducing sugar yield than the non-pretreated biomass (Table 2). The hydrolysis of inexpensive lignocellulosic raw materials results in the less environmental impact when compared to the other physico-chemical pretreatment methods [29]. The resulting hydrolyzed components are more susceptible to the enzymatic hydrolysis. Hence coupling both the pretreatment and subsequent enzymatic hydrolysis process would enhance the sugar yields.

**Table 1**

Lignocellulolytic enzyme activity exhibited by the extracellular protein extract of the bacterial isolate JS-C42. Enzymatic hydrolysis was represented as mean value of triplicate experiments with standard error mean.

Name of assay	Enzyme activity ( $\text{IU mL}^{-1}$ )
FPU activity	$30.71 \pm 0.2$
Endoglucanase	$18.8 \pm 0.4$
Exoglucanase	$27.4 \pm 0.07$
Cellobiohydrolase	$7.42 \pm 0.09$
$\beta$ -Glucosidase	$5.02 \pm 0.12$
Xylanase	$0.34 \pm 0.06$
Lignolytic assay	$0.06 \pm 0.007$

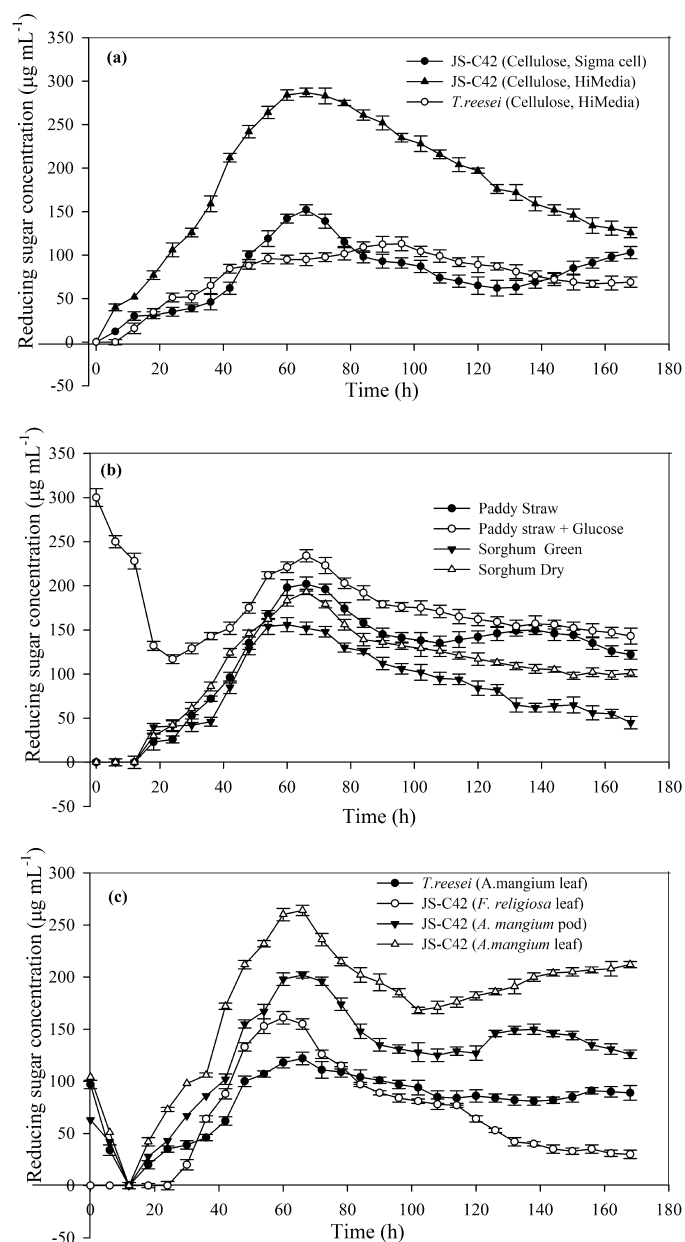
**Table 2**

Saccharification effect on pretreated and non-pretreated biomass substrates exhibited by the bacterial isolate JS-C42.

Plant biomass	Saccharification (%)
Paddy straw pretreated	77.5
Paddy straw non-pretreated	44.3
Sorghum straw pretreated	70.8
Sorghum straw non-pretreated	48.6
Acacia leaf pretreated	78.1
Acacia leaf non-pretreated	65.4

### 3.6. Cellulolytic assay on different plant biomass

Cellulose metabolism by the enzymatic activities of JS-C42 on different substrates were given in Fig. 2a–c. The cellulolytic



**Fig. 2.** Reducing sugar level released by the enzymatic saccharification by the JS-C42 bacterial isolate when grown on: (a) different commercially available cellulosic substrates and *T. reesei* was used for the comparison; (b) agricultural crop materials; (c) steam pretreated plant biomass such as leaves and pods of *A. mangium* and *F. religiosa* leaves.

microbial inoculum was grown on medium with cellulose and degradation of cellulose initiated immediately by the metabolic enzymes secreted by them. Beyond the lag phase after 6 h incubation, the polymeric cellulosic substrate (Cellulose, HiMedia) was consumed at a faster rate, indicating an exceptionally high rate of degradation of cellulose by the isolate JS-C42 and it had been highly efficient when compared to the cellulolytic activities of *T. reesei*. However the breakdown pattern of Sigmacell was slow when compared to the HiMedia cellulose. The cellulolytic isolate JS-C42 achieved the maximum cellulolytic action between the periods of 54–78 h of incubation. The maximum sugar content released from HiMedia cellulose and Sigmacell cellulose by JS-C42 was observed at 66 h of incubation with  $287 \pm 9$  and  $152 \pm 8 \mu\text{g mL}^{-1}$  reducing sugar content respectively (Fig. 2a).

Culture supernatants of cellulolytic bacterial isolate JS-C42 were analyzed for reducing sugars, which began to accumulate during the growth on different agricultural biomass; paddy straw, paddy straw with glucose, dry and green sorghum stubbles with the high level of enzymatic saccharification between the periods of 54–78 h after inoculation. The maximum enzymatic breakdown of lignocellulosic biomass by JS-C42 and the level of reducing sugar concentrations were observed as  $198 \pm 9$  to  $202 \pm 8$ ,  $154 \pm 8$  to  $156 \pm 7$  and  $183 \pm 6$  to  $193 \pm 3 \mu\text{g mL}^{-1}$  at 60–66 h from paddy straw, dry and green sorghum stubbles respectively. At the end of experimental reactions, the reducing sugars detected as  $122 \pm 5$ ,  $45 \pm 7$  and  $101 \pm 4 \mu\text{g per mL}$  (Fig. 2b), however the quantity was less when compared at 60–66 h of inoculation.

The biologically active cellulase enzyme complex has been produced by JS-C42 in order to utilize the biomasses of tree crops. In case of *A. mangium* pods and leaves, the steam pretreatment released certain level of reducing sugars ( $97 \pm 4$  to  $104 \pm 4 \mu\text{g mL}^{-1}$  and  $63 \pm 3 \mu\text{g mL}^{-1}$  respectively from leaf and pod extract) and all those sugars were utilized by the cellulolytic bacterial isolate JS-C42 within 12 h incubation at  $30^\circ\text{C}$ . Beyond this time, again the reducing sugars started to accumulate in the medium due to the lignocellulolytic action and the maximum sugar content was released during the period of 48–78 h (Fig. 2c). The sugar release pattern was higher when compared to the cellulolytic effect exerted by the *T. reesei*. The sugar content released by *T. reesei* from *A. mangium* leaf was observed maximum at 48 h onwards and maintained almost at a constant level for a period of 168 h. In case of *F. religiosa*, the enzymatic hydrolysis of cellulose was observed from 30 h after incubation, the sugar content was observed maximum from 48–78 h and after 114 h, the sugars were metabolized by the JS-C42 itself and there was a drastic decrease in the reducing sugar level when compared to the sugar content from *A. mangium*.

The microbial metabolism of cellulose is gaining importance in recent years due to applications in the production of cellulosic ethanol preferred over grain ethanol [30]. During plant biomass breakdown into simple sugars, the bacterial isolate JS-C42 was able to utilize all four particulate substrates such as paddy straw, sorghum, leaves of *F. religiosa*, pods and leaves of *A. mangium* in a similar fashion, and there was a significant correlation in the apparent loss of substrate dry weight and simple sugar accumulation during the course of the fermentation process. These results also demonstrated the ability of halotolerant bacterial isolate JS-C42 to degrade complex cellulosic substrates into simpler forms.

The overall trend in reducing sugar release by the bacterial isolate JS-C42 from various plant biomass was almost similar, however each plant biomass differed in the level of sugar release. The uptake of released sugar for the bacterial metabolism is low when compared to the amount of free sugar present in the spent medium. The residual cellulose over the time course experiment denoted there was a gradual decrease in hydrolyzable cellulose content by the enzyme released by the cellulolytic bacteria at the maximum reducing sugar release stage (48–78 h). The enhanced initial growth

in the medium supplemented with 0.03% glucose along with the biomass (paddy straw) showed the cellulolytic bacterial isolate JS-C42 can utilize the initial glucose content and once reaching the threshold level, there is a proportionate rise in the availability of free glucose in the medium over the course of time.

### 3.7. Conversion of reducing sugars into cellulosic ethanol

Production of second-generation ethanol from plant biomass is an advantage over the starch ethanol, due to the high amount of reducing sugars derived from saccharification of cellulosic plant biomass. During the fermentation process, the reducing sugar derived from the biomass of *A. mangium*, was converted into ethanol and the ethanol yield was compared with the maximal theoretical yield for the glucose ( $510 \text{ mg/g}$ ). Concentration of ethanol increased with the time accompanied by the drastic reduction in the reducing sugar level in the fermentation broth. The highest concentration of ethanol production was observed at 42 h in case of sugar derived from *A. mangium* leaves and the detected quantity was  $82.4 \text{ mg g}^{-1}$ . Likewise at 54 h higher level of ethanol ( $65.3 \text{ mg g}^{-1}$ ) was observed for the *A. mangium* pods derived reducing sugars. In case of Ficus leaves, paddy straw and sorghum stubbles, the maximum alcohol content was quantified as 43.1, 63.1 and  $54.5 \text{ mg g}^{-1}$  respectively (Fig. 3). Inoculation of yeast cells at low cell counts in a fermentation medium with high glucose concentration is thought to be inappropriate in alcohol fermentation systems due to the excess yeast growth and multiplication in terms of increase in biomass rather than ethanol fermentation in early stage [31]. Hence the conversion of reducing sugars into ethanol during fermentation was initiated with high inoculum loads of yeast cells.

The gross energy value of ethanol produced from the different steam pretreated biomasses at laboratory scale were 2.21, 1.75, 1.16, 1.69, 1.46 MJ/kg respectively for the *A. mangium* leaves, *A. mangium* pods, Ficus leaves, paddy straw and sorghum stubbles. The ethanol-equivalent energy consumption from pretreatment of biomass to ethanol production was equivalent to 0.81 MJ/kg (based on the operating parameters of high-pressure steam vessel and fermentor). The pseudo-net energy value of ethanol produced from the steam pretreated *A. mangium* leaves, *A. mangium* pods, Ficus leaves, paddy straw and sorghum stubbles were 1.39, 0.94, 0.34, 0.88, 0.65 MJ/kg of the biomass respectively. The leaves of *Acacia* showed high net-pseudo energy value and Ficus with less net energy value of ethanol yield. It also suggests though a significant

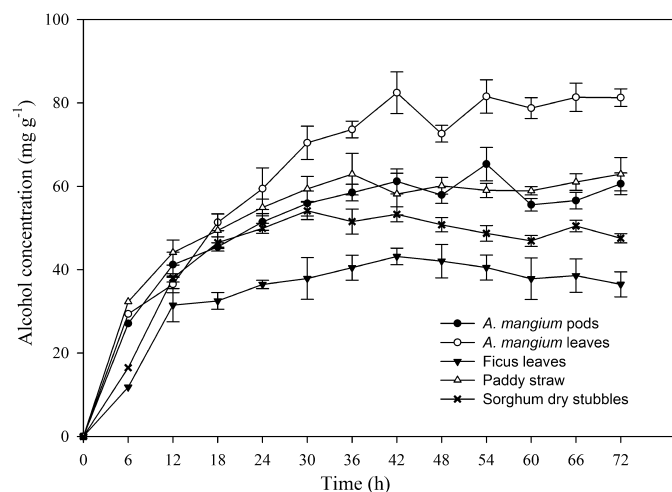
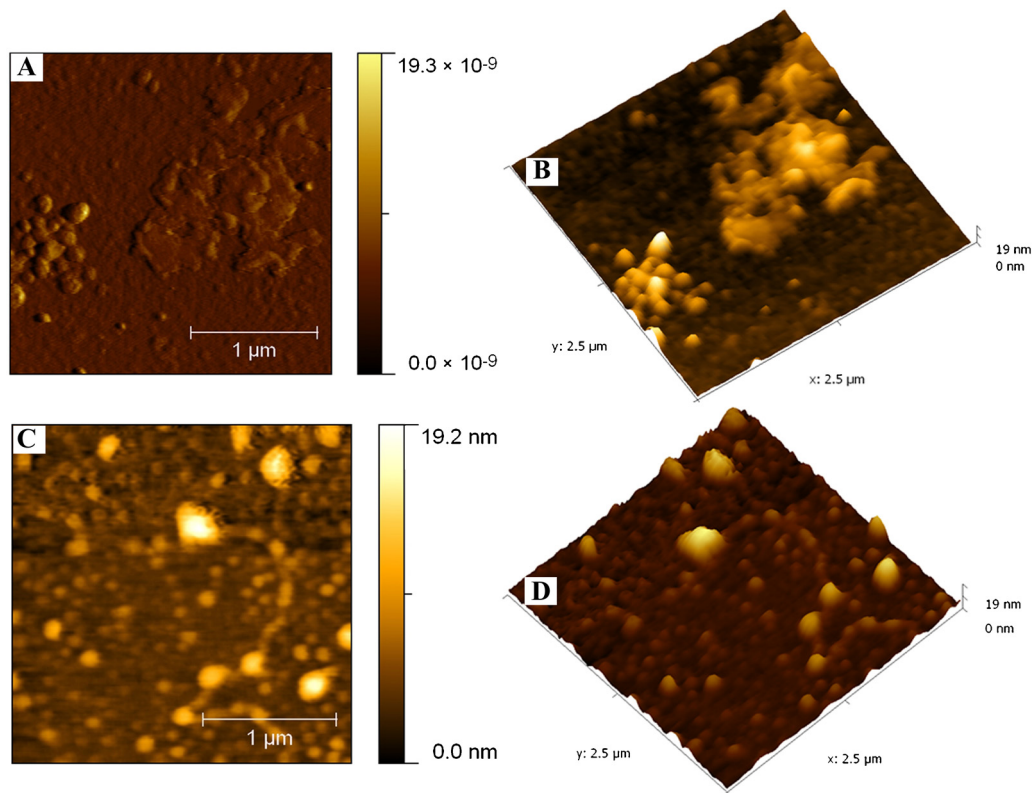


Fig. 3. Ethanol production profile of *S. cerevisiae* MTCC 170 from the reducing sugars released by the *Isoptericola* sp. JS-C42.



**Fig. 4.** Non contact mode atomic force microscope image analysis of the *Isoptericola* sp. JS-C42. A and B denote the 2D and 3D images of bacterial cells colonized on the surface of the Sigmacell cellulose substrate. C and D denote the 2D and 3D surface topography of JS-C42 spread on a glass cover slips without cellulose substrate.

level of energy is consumed for the lignocellulosic ethanol production from the steam pretreated biomass, it is an indispensable source of alternative fuel energy.

The strong crystalline structure of cellulose, complex hemicelluloses and lignin contents of the crop residues and tree leaf litters limits accessibility of plant biomass to hydrolytic enzymes [32]. However the marine bacterial isolate JS-C42 showed the efficient lignocellulolytic ability to release the reducing sugars from steam pretreated biomass due to the increase in the accessible cellulosic surfaces for the enzymatic actions. Thus the synergistic action of cellulolytic enzymes with the steam pretreated substance helps in the production of cellulosic ethanol by the substantial release of simple reducing sugars. This study enumerated the release of reducing sugars from the lignocellulosic materials by the subsets of lignocellulolytic enzymes secreted by the bacterial isolate JS-C42 without any external input of commercial enzymes.

### 3.8. Atomic force microscope image analysis of *Isoptericola* sp. JS-C42

The average diameter size of the bacterial cells grown on tryptic soy broth without cellulose was  $0.117 \mu\text{m}$ . When the cells grown on Sigmacell cellulose, they were colonized on the surface of the cellulose substrate and they appeared plumpier than the cells grown on tryptic soy broth. The average diameter of cells grown on the microcrystalline cell surface was  $0.150 \mu\text{m}$ . Atomic force microscope image analysis of 12 h grown *Isoptericola* sp. JS-C42 in the present study revealed the mycelial form (Fig. 4) with embedded cocci shaped cells appearing like beads on a string arranged in an irregular pattern. The diameter of the cells in the mycelium ranged  $0.107$ – $0.264 \mu\text{m}$ . The behavior of bacteria on cellulosic surfaces with different media components as well as their interaction and adherence is of prime importance in the enzymatic breakdown

of cellulose for their current and future applications like ethanol production. Members of *Isoptericola* are actinomycetes with a well-developed primary mycelium that broken into small cocci and rod like structures [33] and the atomic force microscope image of JS-C42 also showed the presence of primary mycelia form with budding cocci form. The atomic force microscope phase imaging suggested that individual bacterial cells JS-C42 were adsorbed onto the surface of a cellulose substrate.

## 4. Conclusion

To our knowledge the present study was the first report by the halotolerant bacterial isolate *Isoptericola* sp. JS-C42 in displaying an efficient enzymatic saccharification of plant biomasses and the released reducing sugars can be successfully converted in to ethanol. To decrease the cost of commercial cellulolytic enzymes and the hazardous effect of harsh pretreatment of lignocellulose by the chemical processes, the marine derived bacterial isolate *Isoptericola* sp. JS-C42 is an option to consider an alternate means to produce the economical and environmental friendly saccharifying sugars from lignocellulosic substrates for the bioethanol production. This data also suggested that a significant proportion of the resident cellulolytic microorganisms of marine sediment remain poorly characterized and exploring those microorganisms is a boom to the applications related biofuel research and development.

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