

# **Cellulase: Critical enzyme of biofuel industry – A sea water based approach**

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Lignocelluloses are the most abundant biomass available on earth with immense potential to meet global energy demands in sustainable manner. Few key factors are involved in order to accomplish the same and cellulases play a pivotal role in this task. Cellulases are critical enzymes in biofuel and food industries. Several bacterial and fungal species have been reported to be the cellulase producer using different cellulose sources. Utilization of fungal strains has some advantages over bacterial strains as cellulase producers. Likewise there are many reports published which studied the utilization of cellulases for saccharification of bioresources for production of biofuels. However, all these works have been carried out using fresh water as a source of medium. Recent public threats on fresh water depletion signify the exploration of non-freshwater medium for the production of biofuels. Among the non-freshwater sources, seawater is the best source to be studied as medium for biomass conversion due to its abundant availability in India. Utilization of halotolerant microorganisms capable of producing salt tolerant enzymes will be a major breakthrough in this field as they can tolerate high salt levels and ionic liquids better than current fungal cellulases. Further, there will be advancement in use of sea and brackish water for biomass conversion. The present study focuses on isolation and screening of both bacterial and fungal strains from coastal zones of Odisha, capable of producing halotolerant cellulases. All the isolates were screened for their cellulolytic ability and their enzymatic properties were characterized using soluble cellulose sources in fresh as well as in seawater. The potent bacterial and fungal strains were characterized for different parameters like optimal pH and temperature of enzyme action along with effect of metal catalyst. For the bacterial cellulases the optimal pH was found to be at physiological pH whereas in case of a potent fungal cellulase isolated from paddy field was found to be stable over a wide range. Optimal temperature of enzyme action in case of bacterial cellulases was recorded to be between 45-55°C whereas in case of its fungal counterparts was found to be thermostable i.e. stable at 85°C. Manganese ions used as cofactor played outstanding role in enhancing the potential enzyme activity manifold compared to the one without cofactors. All the enzymes from different isolates have been partially purified and future work include their complete purification and active site determination for further studies and implementation on larger scale.



# **Cellulase: Critical enzyme of biofuel industry-A sea water based approach**

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**Odisha, India**

# Cellulosic Ethanol – Solution for World's problem

- Second generation biofuel
- Cellulose Sources Do not cause Food Vs Fuel crisis
- India's first biofuel powered ship hit the seas during second week of February
- India: Assam – 110 Million Pound Joint Venture cellulosic ethanol project (Chempolis Ltd., Finland and Numaligarh Refinery Ltd, India (Starting from 2019) with coproduction of acetic acid and furfural
- Bamboo based facility – 49,000 metric tons of ethanol annually (56 million litres) (Hydrocarbon vision 2030 of North East



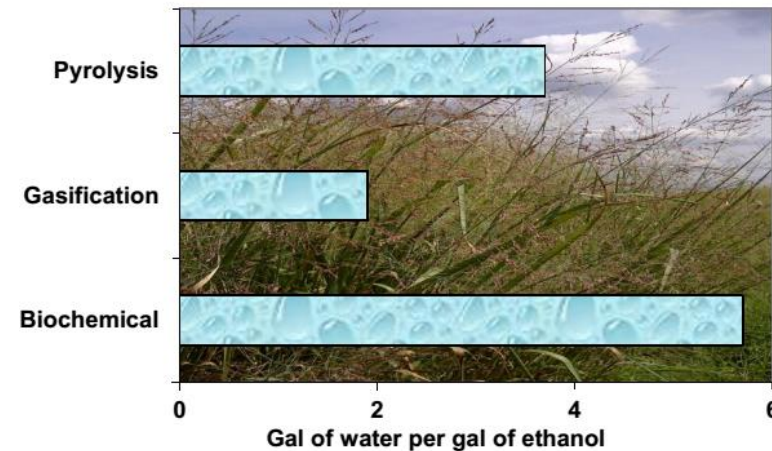
- Global second generation biofuel market – estimated to growth at a CAGR of 49.4% over 2014-2020
- Reach 23.9 Billion US dollar



# Bioethanol's thirst for water

- ✓ 6 to 10 litres of water utilized / litre of ethanol produced
- ✓ 5.8 litre water in 1998, 4.2 litres of water in 2005
- ✓ Irrigation - Growing biomass (corn, sugarcane, and other plants)
- ✓ Switch grass – drought tolerant – but may require water to increase yield and require water for processing
- ✓ Corn ethanol consumes 85 litres to 330 litres of water per 1 litre of ethanol (the range is due to different irrigation requirements),
- ✓ Gasoline consumes 14 to 27 litres, and switchgrass consumes 8 to 34 litres (the range is due to different production technologies).
- ✓ Production cost of bioethanol will be reduced from Rs. 45 per litres (2012) to Rs. 29 / litre (2020) – Production will get increased

## Cellulosic biofuel - Biorefinery



1.63 litre of ethanol = 1 litre of petrol in terms of calorific value and energy density

1 gallon = 3.78 Lit

Dr. R. Jayabalan, NIT Rourkela, 26 Feb 2016

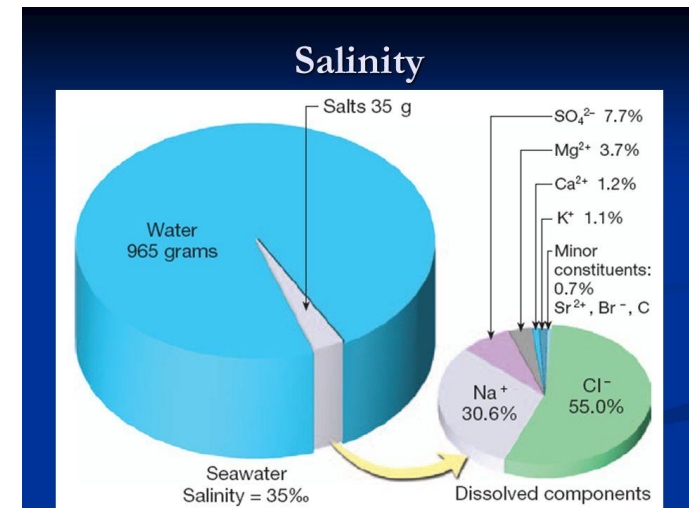
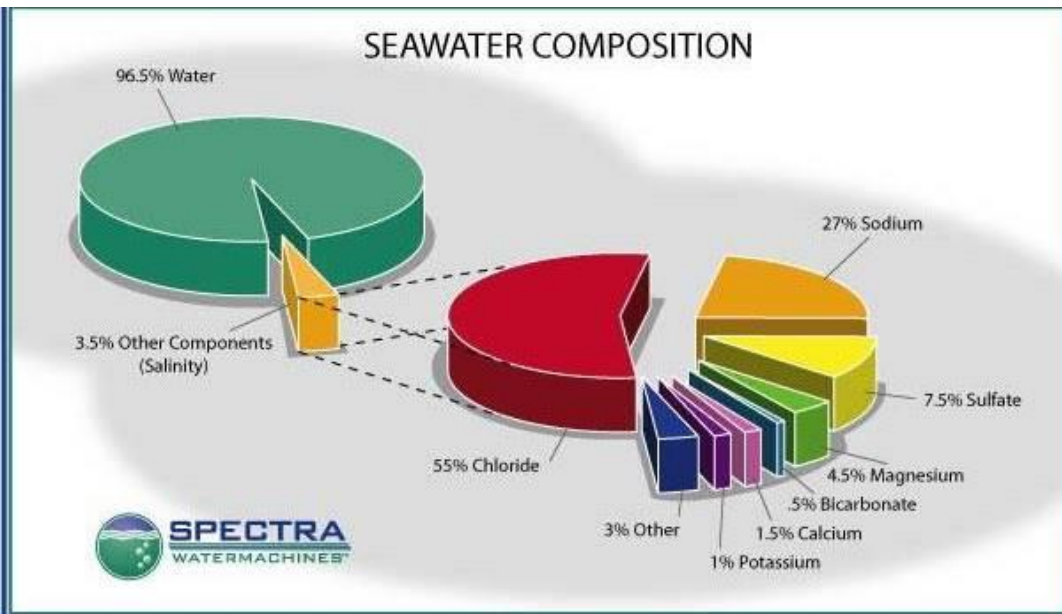
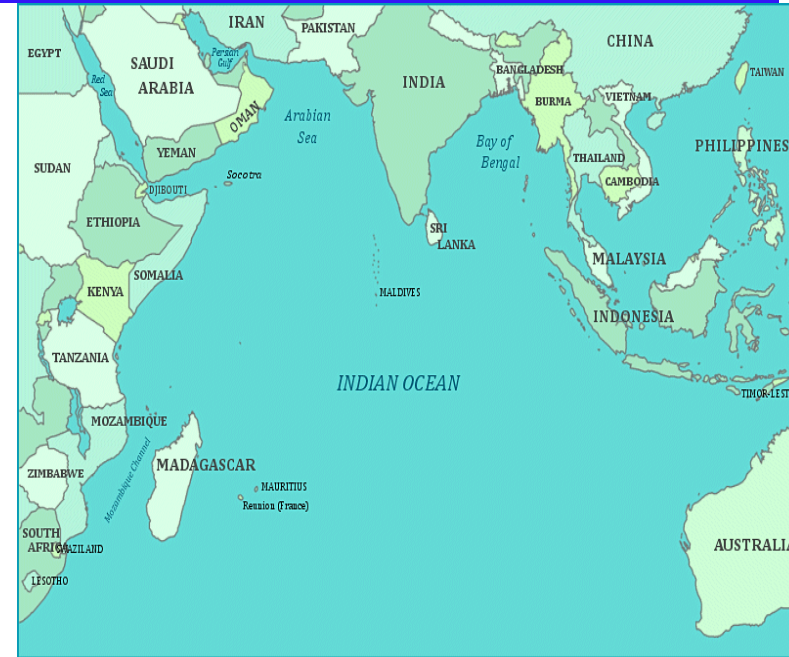
Bala P. Lingaraju, Joo-Youp Lee, Y. Jeffrey Yang. Process and utility water requirements for cellulosic ethanol production processes via fermentation pathway. Environmental progress and sustainable energy, 32(2) DOI 10.1002/ep

NIRE, Kapurthala

Argonne national laboratory

# Why not sea water??

- Research reports available for marine microbial enzymes, growing microorganisms in sea water
- Why not sea water used for processes in ethanol production?



[glossary.periodni.com](http://glossary.periodni.com)

Dr. R. Jayabalan, NIT Rourkela, 26 Feb 2016

<http://www.barefootgypsy.com/images/portfolio/SWM-ppt1-BG.jpg>

# Need of Halotolerant Hydrolytic Enzymes

- ✓ Acid or alkali treated lignocellulosic biomass is neutralized resulting in high salt concentration or osmotic pressure where enzymes lose their activity, halotolerant enzymes find use under such conditions.
- ✓ Halotolerant enzymes have polymer degrading ability at low water activity.
- ✓ Use of enzymes in organic solvents increases solubility of non-polar substrates and eliminate microbial contamination in reaction mixture, enzymes from halophiles or halotolerant organisms are thought to play an important role in such systems.
- ✓ Use of halotolerant enzymes helps to reduce the need for high temperature and pH neutralization for pretreated biomass before fermentation.

# Advantages of Saline System

## Fresh water Management

- ✓ Using seawater for marine algae cultivation on arid land masses reduces total water intake for energy crop production.
- ✓ Some microalgal species can thrive in seawater/saline ground water unsuitable for conventional crops.
- ✓ Using seawater or saline ground water for pretreatment, saccharification and fermentation will result in water management.
- ✓ The use of halophilic/halotolerant algae can greatly reduce the amount of water required for biofuel production.



# Objectives

## Bacterial Cellulase

- To isolate the cellulase producing bacteria from Gopalpur, Odisha (Saline region)
- To compare the enzyme production in freshwater and sea water

## Fungal Cellulase

- To optimize the conditions for the cellulase production by *Fusarium subglutinans* MTCC 11891 (isolated from rice field and deposited to MTCC)
- To compare the production of cellulase in Mandel's media and sea water media



# Isolation and screening of halotolerant bacterial cellulase from Gopalpur, Odisha

Congo red agar plates



**GSI**



**GS2**



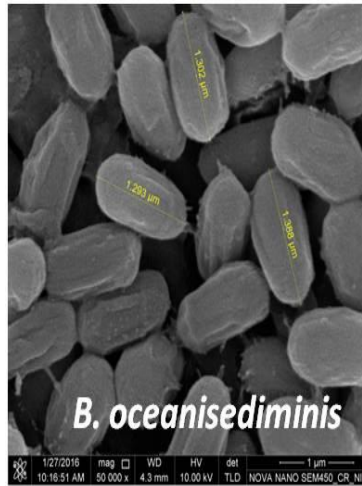
**GS3**



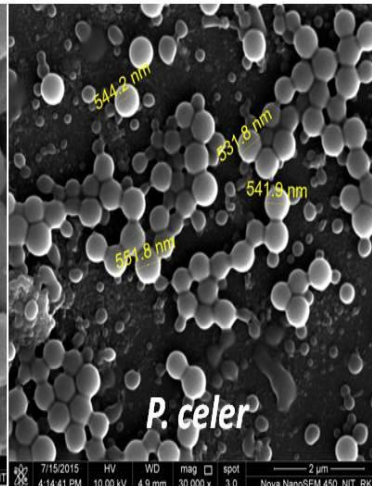
**GS4**



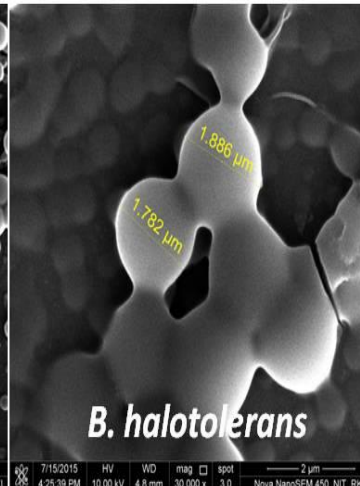
**GS5**



*B. oceanisediminis*



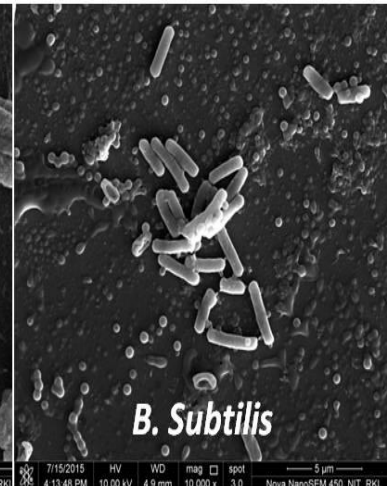
*P. celer*



*B. halotolerans*



*P. aeruginosa*



*B. Subtilis*

*Bacillus  
oceanisediminis*

*Pschirobacter  
celer*

*Bacillus  
halotelarans*

*Pseudomonas  
aeruginosa*

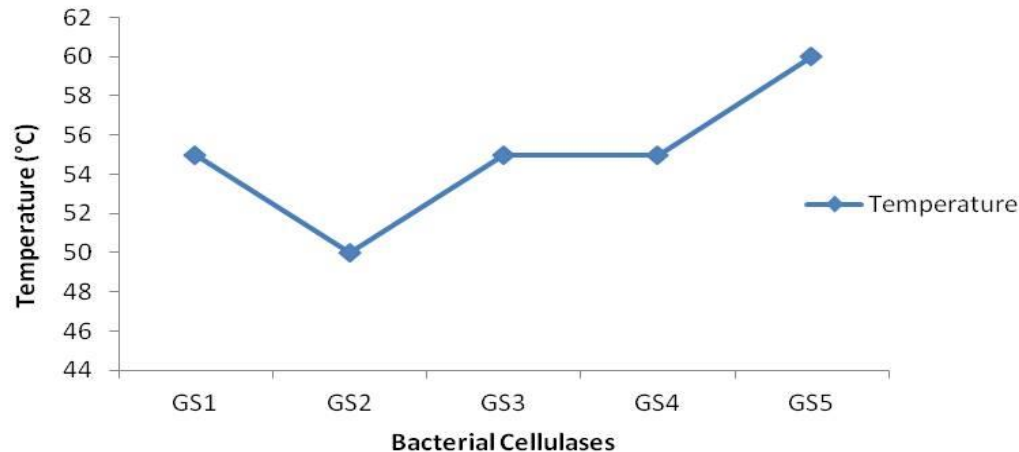
*Bacillus subtilis*

SEM images

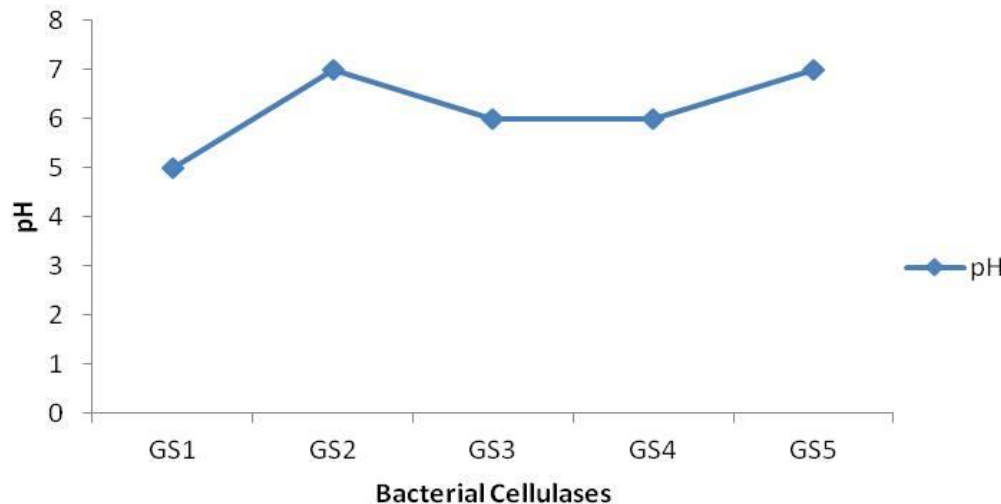
Identification through 16srRNA technology

# pH and Temperature optimization

Optimal Temperature of action

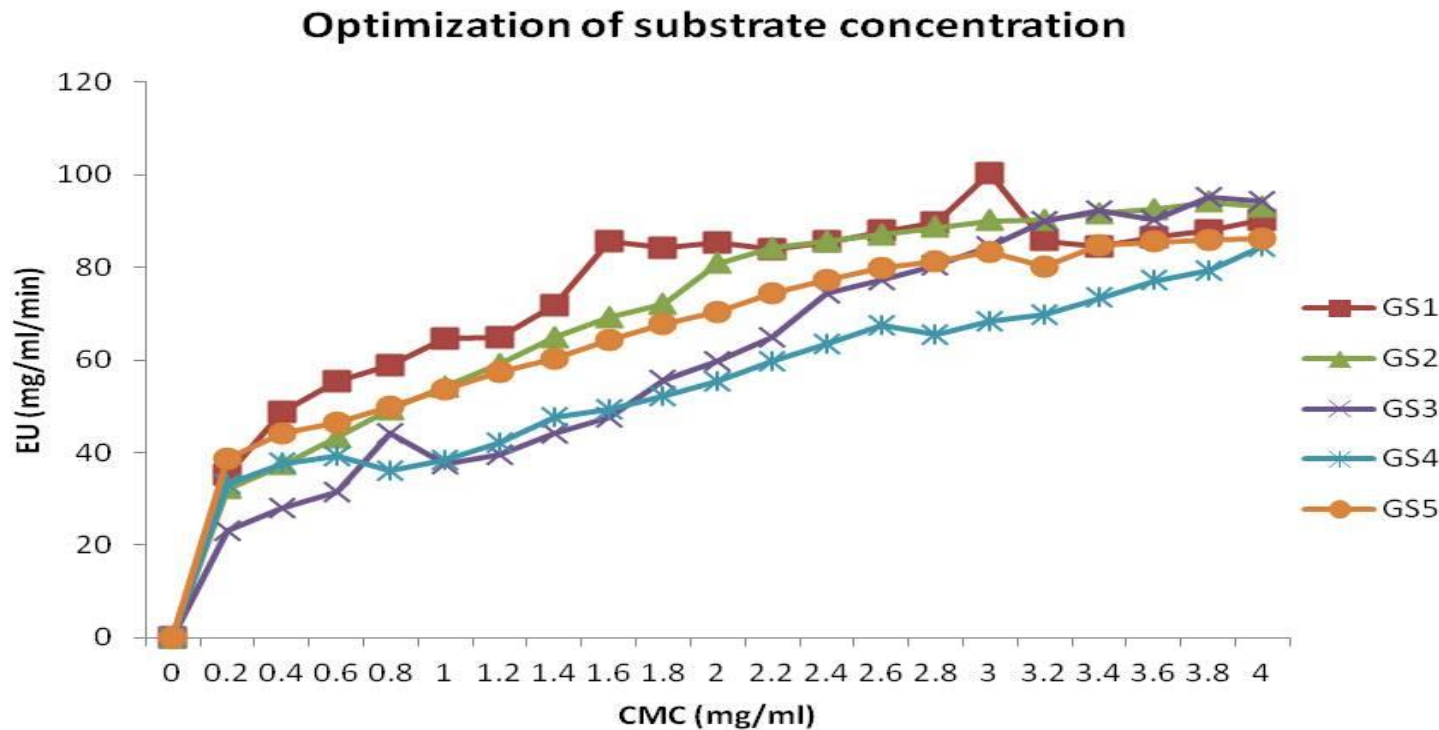


Optimal pH of action



- ❖ Selected cellulose digesting bacteria were cultured on **enzyme production media** at 37°C, 150rpm at pH 7.4-7.8 for 5 days.
- ❖ Cell free supernatant obtained after centrifugation at 5000 g for 15 min at 4°C was stored as crude enzyme extract at 4°C.
- ❖ Determination of enzyme activity is measured using methods suggested by International Union of Pure and Applied Chemistry (IUPAC) (Ghose, 1987).
- ❖ Crude cellulase was characterized for determination of its optimal pH and temperature with CMC as substrate for hydrolysis.

# Optimization of substrate concentration

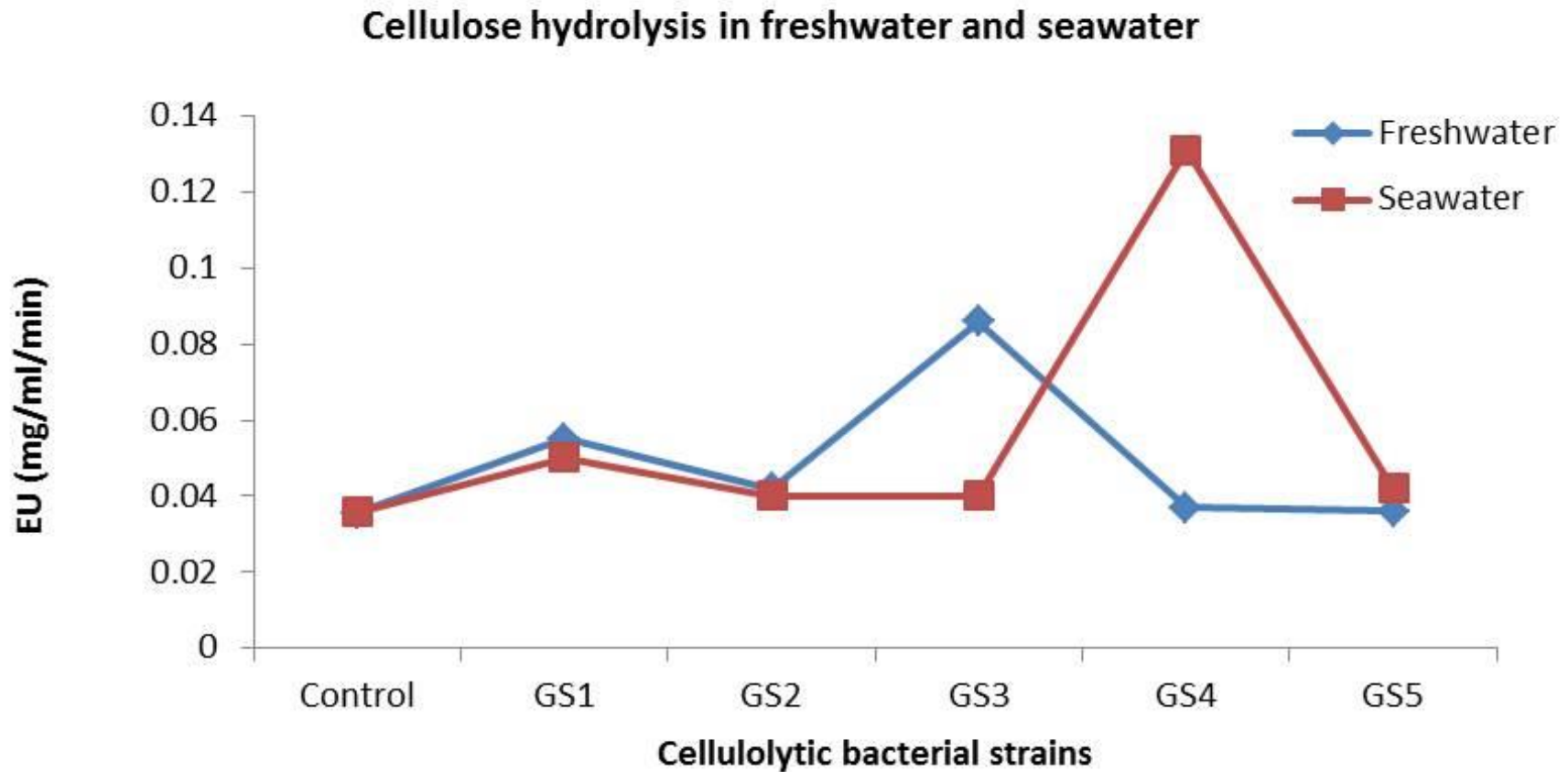


- ❖ The values of the Michaelis constant ( $K_m$ ) and the maximum velocity ( $V_{max}$ ) were obtained by measuring the rate of hydrolysis of CMC under optimal temperature ( $50^\circ\text{C}$ ) and pH (7.0) conditions at substrate concentrations ranging from 0.2 to 4 mg/ml (treatment period 15 mins).
- ❖ Values for  $K_m$  and  $V_{max}$  were determined from Michaelis-Menten kinetics (around 2.0 mg of CMC / mL of buffer and  $1.942 \times 10^{-7}$  to  $7.271 \times 10^{-7}$   $\mu\text{M/mL/min}$ ) (Sorenson's buffer at pH 7.0)
- ❖ Optimization of substrate concentration results in augmentation for proper enzyme activity for maximum conversion.

# Concentration of metal ions in used sea water (AAS tudy)

Element	Percent
Oxygen	85.84
Hydrogen	10.82
Chloride	1.94
Sodium	1.08
Magnesium	0.1292
Sulphur	0.092
Calcium	0.04
Potassium	0.04
Bromine	0.0067
Carbon	0.0028

# Cellulose hydrolysis in freshwater and seawater



- ❖ Cellulase activity was determined under optimal temperature and pH at specific substrate concentration independently in fresh water and seawater in order to determine the ability of cellulase activity in filtered and autoclaved seawater based systems.
- ❖ *P. aeruginosa* show enhanced cellulolytic potential in seawater as compared to the other four strains.
- ❖ Co-factors plays major role in enhancing activity of enzyme. Future work includes standardization of enzyme activity in seawater in presence of co-factors.

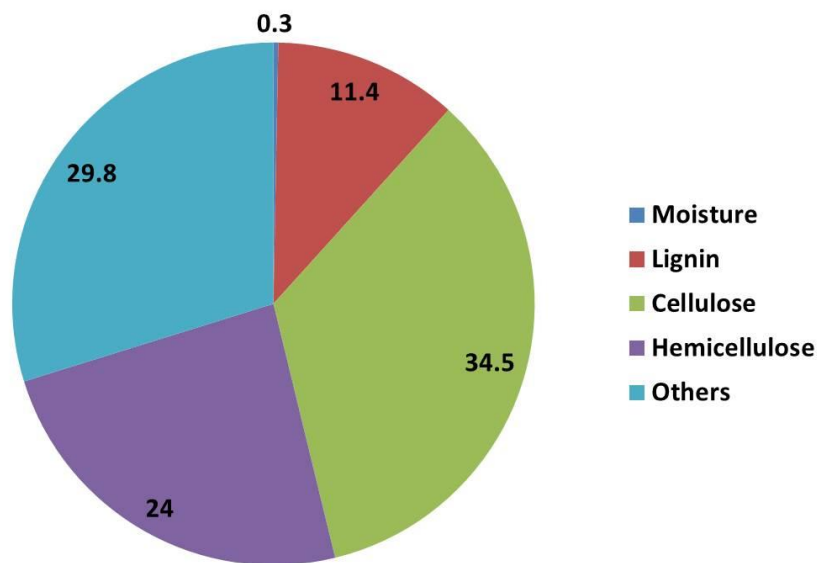


# Optimization of cellulase production by *Fusarium subglutinans* MTCC 11891 using sea water as production medium



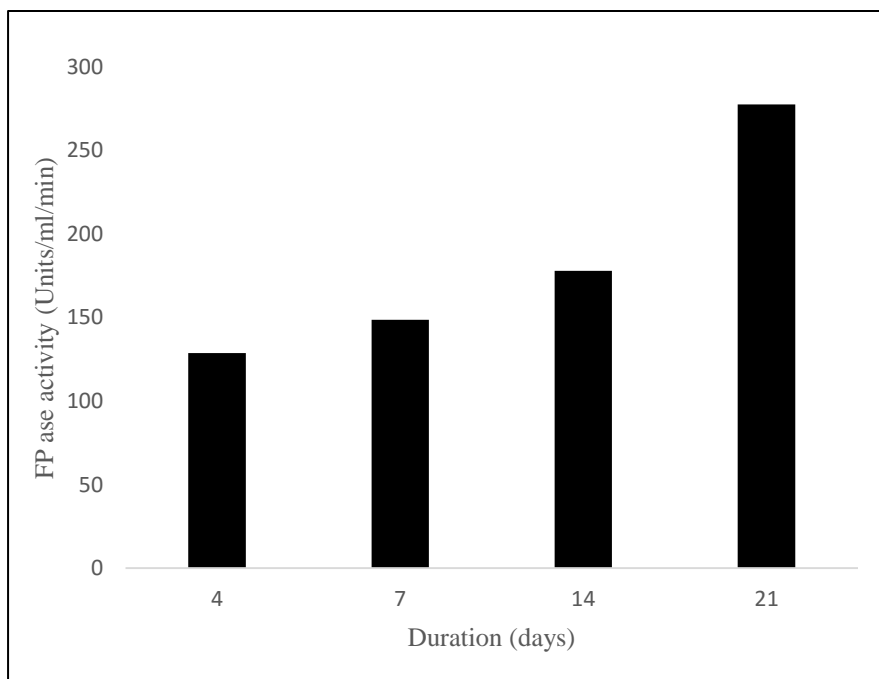
- ✓ Isolation of fungus from rice field – out of three fungus isolated – *F. subglutinans* shown maximum cellulolytic ability in sea water
- ✓ Fungal isolate was identified as *Fusarium subglutinans* (MTCC 11891) and deposited in MTCC & GB of IMTECH, India

# Pretreatment of Rice straw (biomass)

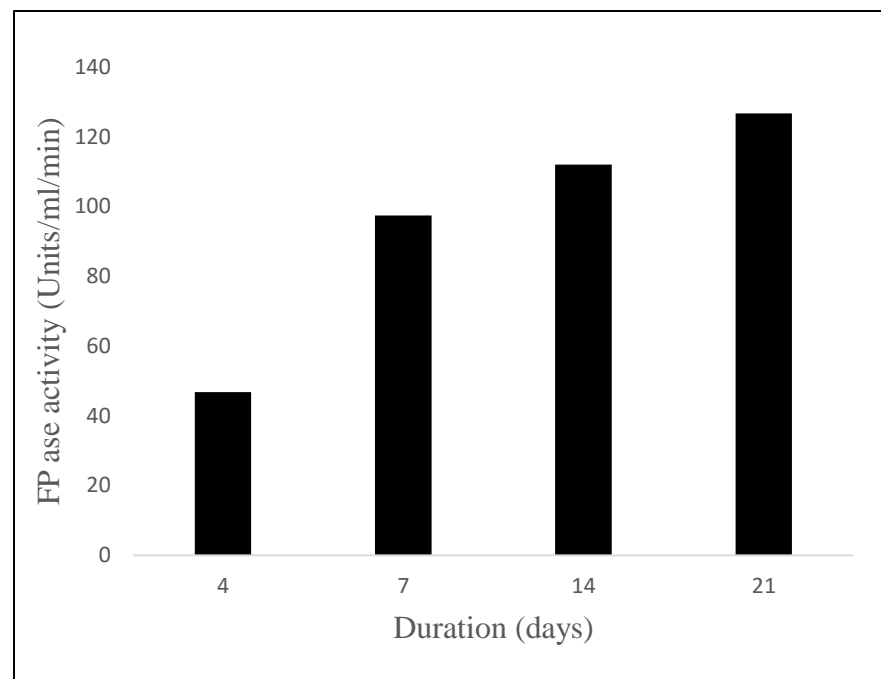


- ❖ Being abundant in the region and due to lack of any substantial use, rice straw is the lignocellulosic biomass.
- ❖ Biomass is pre-treated with 1% NaOH at 121°C at 15 lbs pressure for 20 min.
- ❖ Moisture (3%) and lignin content was determined by Jørgensen et al. (2007).
- ❖ Estimation of cellulose and xylose performed by method suggested by Updegraff (1969).



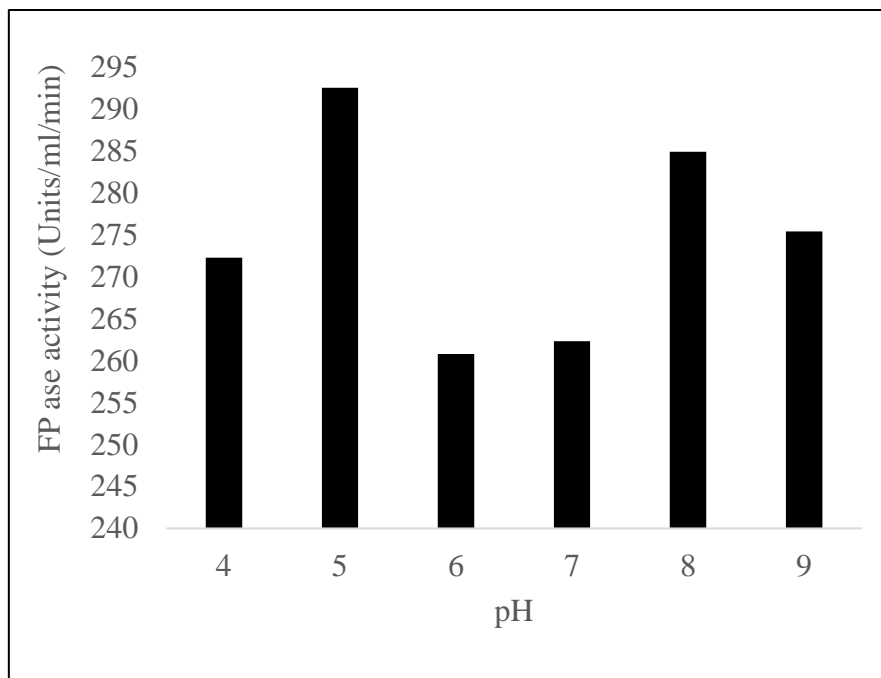


**a) Mandel's media**

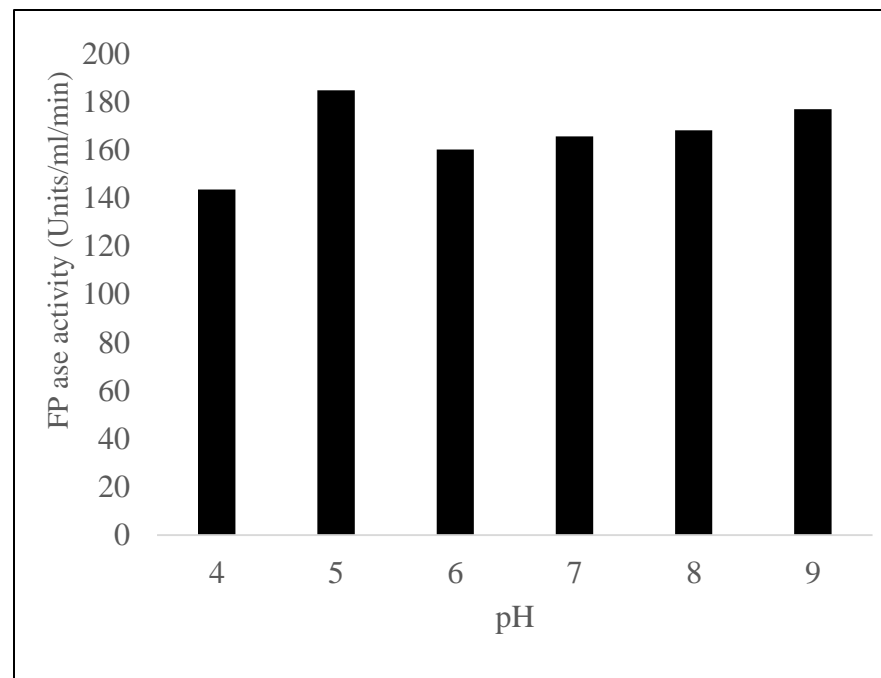


**b) sea water**

**Figure : Cellulase production by *Fusarium subglutinans* MTCC 11891 in shake flask  
50% of enzyme activity is retained in sea water**

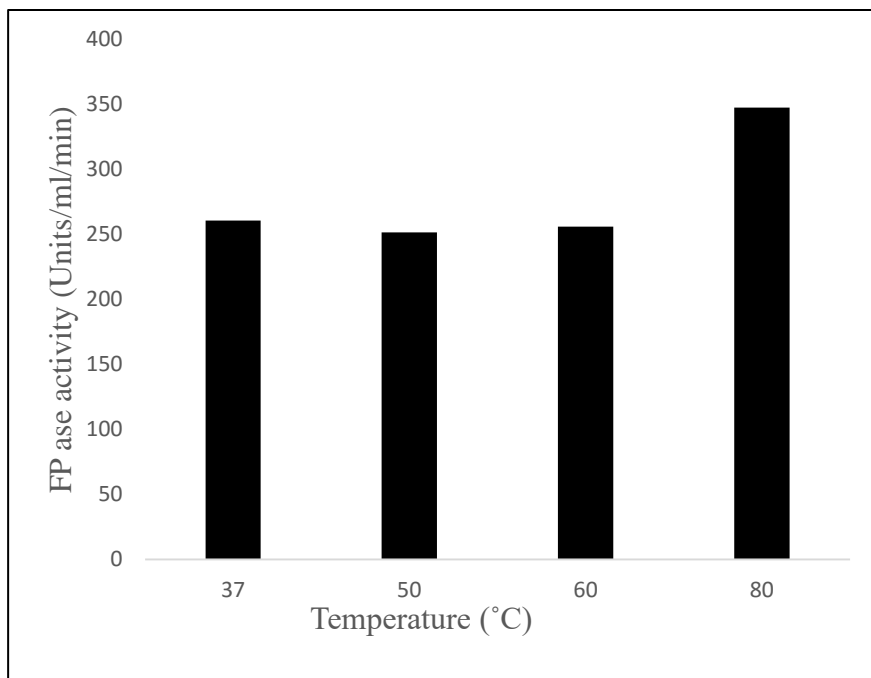


**a) Mandel's media**

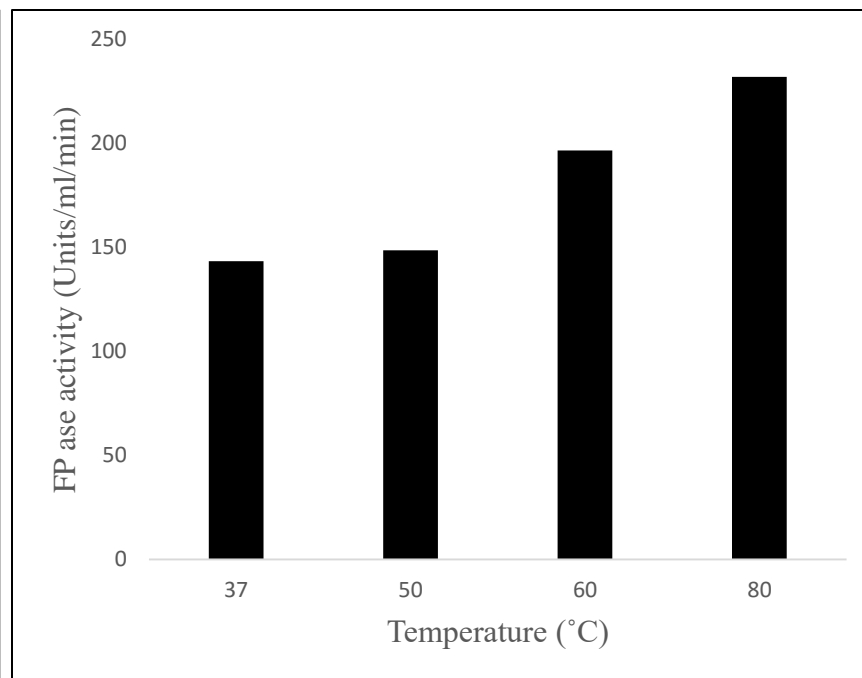


**b) sea water**

**Figure : Effect of pH on cellulase activity**

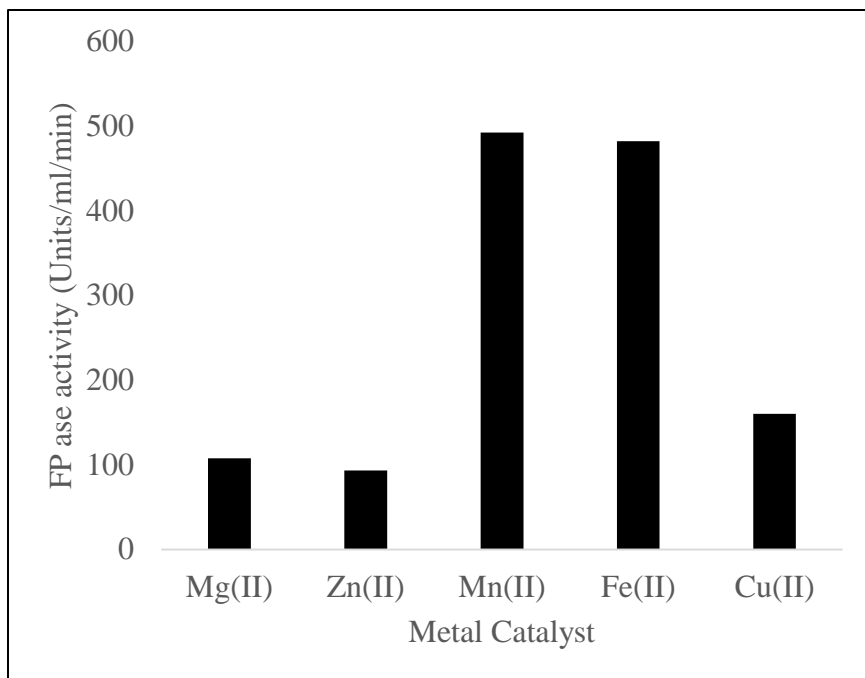


**a) Mandel's media**

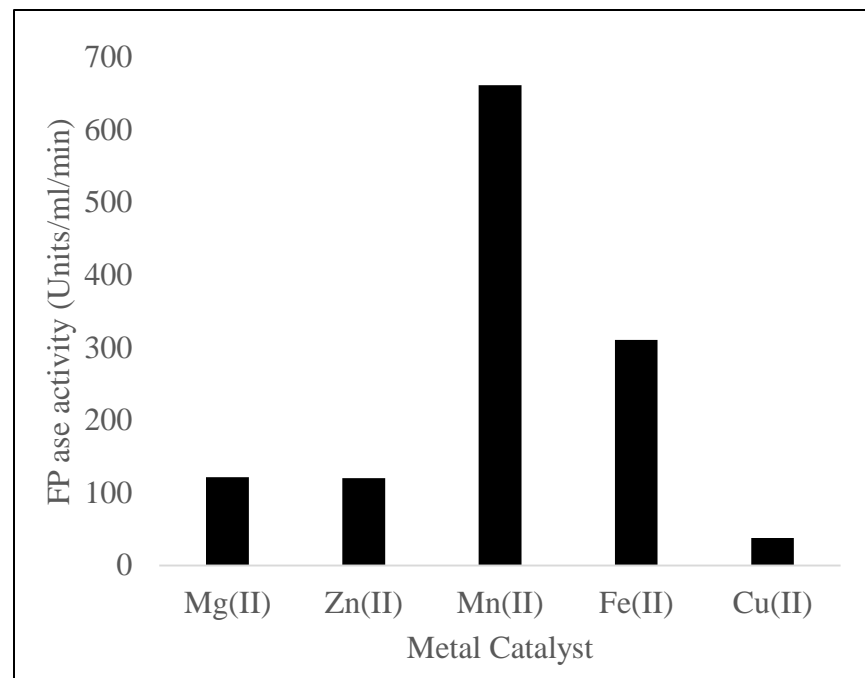


**b) sea water**

**Figure : Effect of temperature on cellulase activity**



**a) Mandel's media**



**b) sea water**

**Figure : Effect of metal ions on cellulase activity**

# Partial purification of cellulase using anion exchange chromatography (DEAE sepharose column)

Concentration of NaCl (M)	FPase activity ( $\mu\text{M}/\text{ml}/\text{min}$ )
0.2	35.32
0.4	49.08
0.6	52.73
0.8	298.68
1	273.12
1.2	45.62
1.4	40.73
1.6	38.95

80% ammonium sulfate precipitation  
Dialysis – Membrane 14kD,  
50 mM Tris HCl buffer pH 7.5  
Column elution buffer – 50 mM Tris HCl buffer pH 7.5  
Eluted using gradient NaCl (0.2 to 1.6 M)

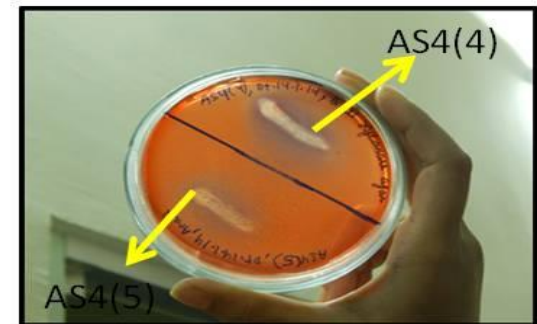
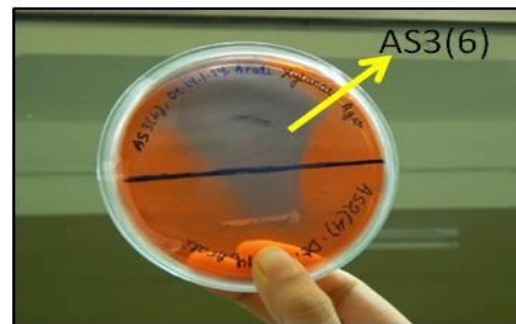
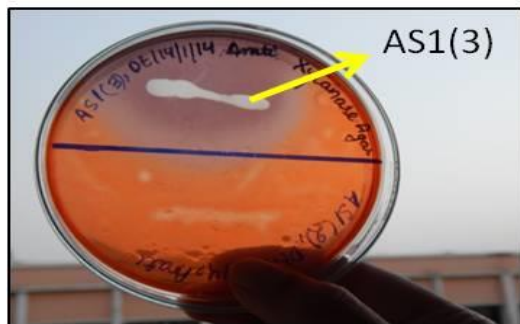
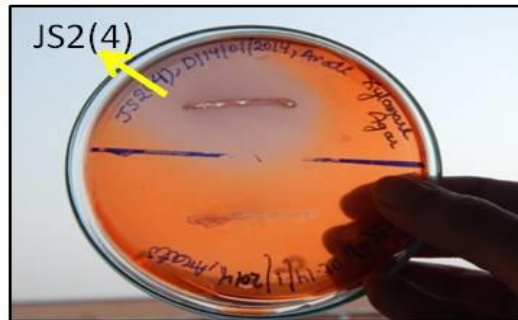
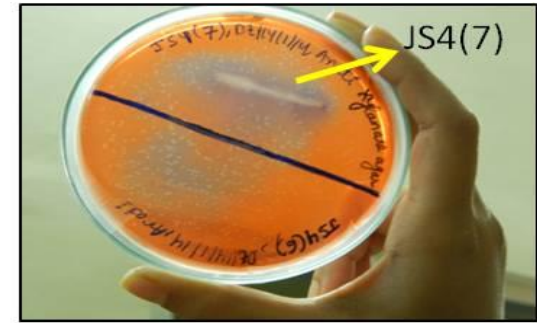
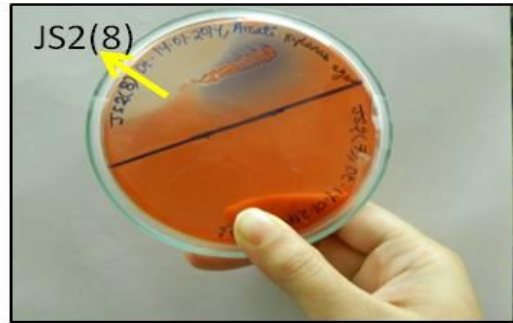
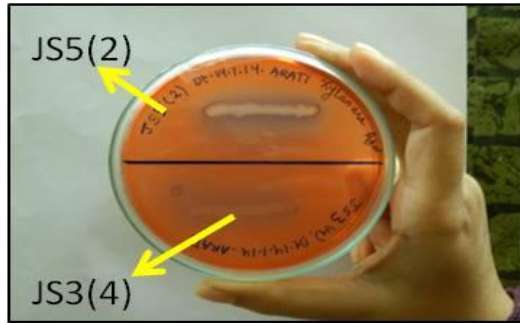
Fractions with maximum cellulase activity were pooled out for further purification and studies

# Importance of Work

- ✓ Novel enzymes of halophilic origin will reduce the dependence on fresh water for biofuels production.
- ✓ Halotolerant enzymes will reduce the cost of neutralization for pretreated biomass before fermentation.
- ✓ Optimization of fermentation conditions in saline environment will be a revolutionary step in the field of fermentation technology.

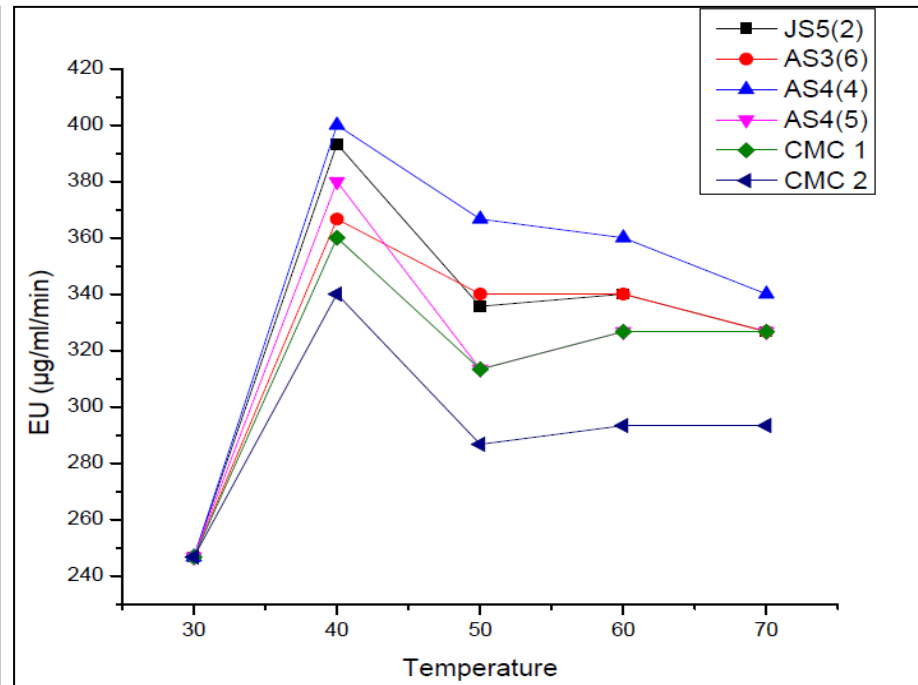
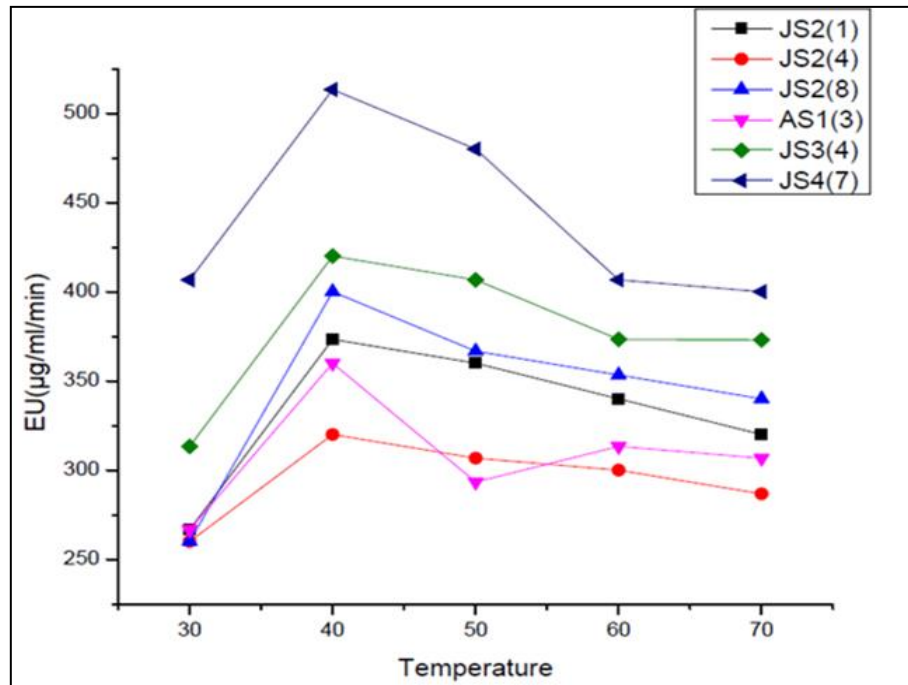
# Xylanase: the collaborative hand

Source: Kitchen waste (Hemicellulose materials)

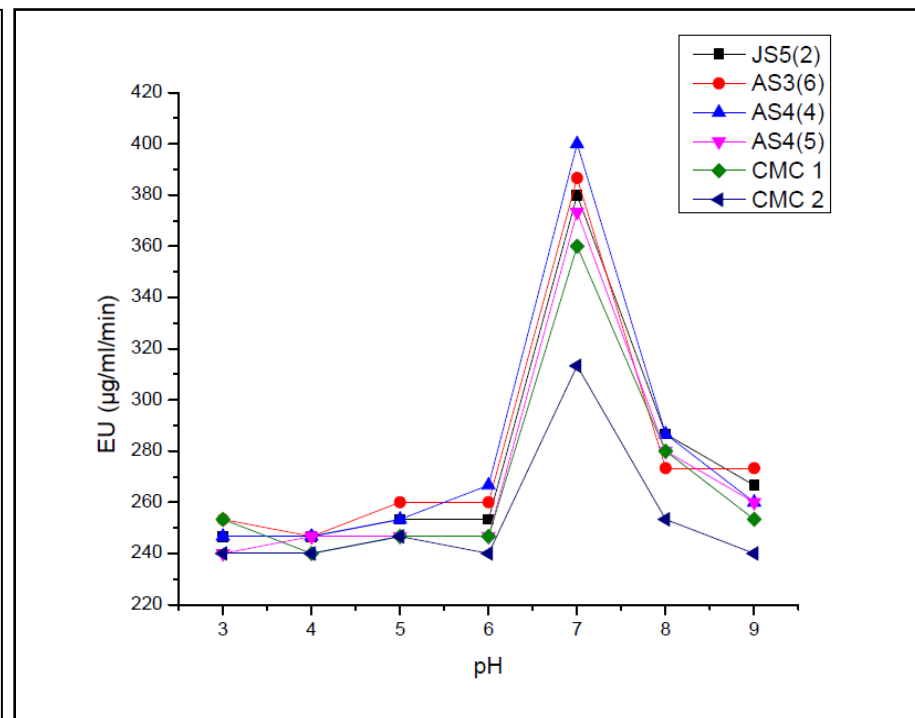
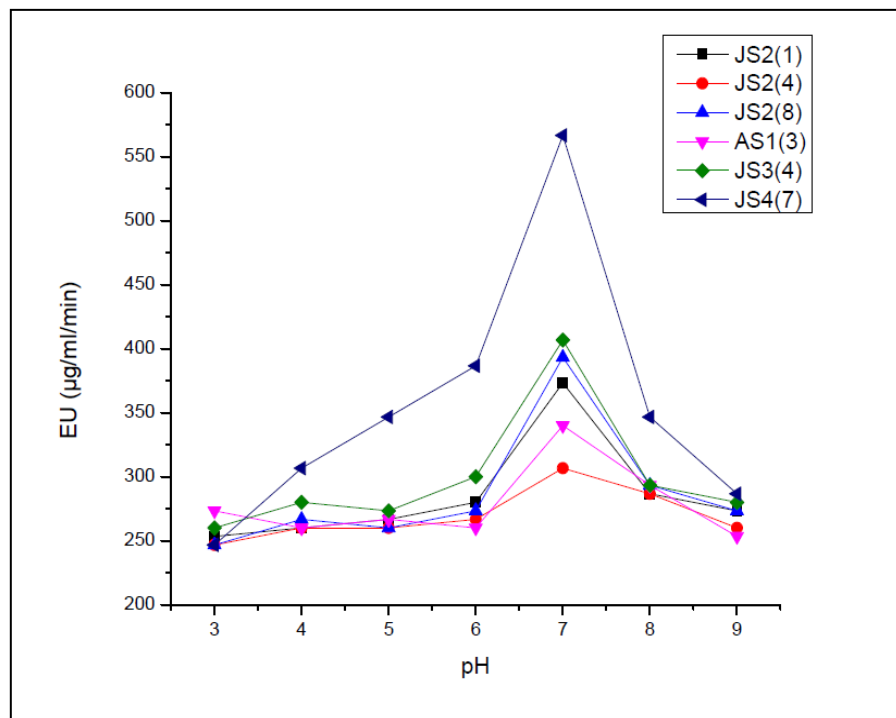




# Optimal Temperature of Xylanase Activity



# Optimal pH of Xylanase Activity



# Km and Vmax

Best one

Substrate (mg/ml) concentration	Measured velocity ( $\mu\text{g/ml/min}$ )
0	0
0.2	35.25
0.4	48.713
0.6	55.466
0.8	58.873
1	64.7
1.2	64.91
1.4	71.733
1.6	85.733
1.8	84.1
2	85.446
2.2	83.81
2.4	85.506
2.6	87.793
2.8	89.713
3	100.3
3.2	85.806
3.4	84.453
3.6	86.586
3.8	88.006
4	90.14

**$V_{\text{max}} = 83 \mu\text{g/ml/min}$**

**$K_m = 0.25 \text{ mg/mL}$**

# Acknowledgement



Indira Dash

- NIT Rourkela
- Dr. Sachin Kumar,  
Organizing Secretary

# Thank you