Consolidated report for R & D Project

[December 2011 to June 2015]

[Extended to June 2015]

Project title

(HC 211)

"Isolation and characterization of hydrogen producing bacteria from North-Eastern states of India, (with special emphasis on Assam and Arunachal Pradesh), for efficient conversion of biomass to hydrogen".

DBT Sanction Order No.& Date: BT/212/NE/TBP/2011 dated December 14, 2011

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Section – A: Project Details

A1. Project Title:- (HC 211): "Isolation and characterization of hydrogen producing bacteria from North-Eastern states of India, (with special emphasis on Assam and Arunachal Pradesh), for efficient conversion of biomass to hydrogen".

A2. DBT Sanction Order No. & Date:- BT/212/NE/TBP/2011, December 14, 2011

A3. Name of Principal Investigator :- Dr. Manabendra Mandal
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A6. Total Cost:

For Tezpur University – Rupees (in lakhs) : 32.31 lakhs (thirty two lakh thirty one thousands only)
For Rajiv Gandhi University- Rupees (in lakhs) : 23.89 lakhs (twenty three lakh eighty nine thousands only)
Total:- Rupees (in lakhs) : 56.2 lakhs (fifty six lakh two thousands only)

A7. Duration: 3 years. (Extended for six months)

A8. Approved Objectives of the Project:

- Collection of Environmental Samples from the different parts of North East India.
- Checking production of hydrolyzing enzymes by the isolated bacterial species.
- Isolation and screening of fermentative hydrogen producer from the environmental samples.
- Identification of the potential strains and optimization of hydrogen production.

A9. Scientific Recommendations made by the Task (If any): Nil

Section – B : Scientific & Technical Progress

B1. Progress made against the Approved Objectives, Targets & Timelines during the Reporting Period (1000 – 1500 words for interim reports; 2500-3500 words for final report; data must be included in the form of up to 3 figures and/ or tables for interim reports; up to 7 figures and/or tables for final report).

Progress

Introduction:-

The global rates of fossil fuel consumption have highly increased during recent decades. This increase has led to serious concern over finding alternate energy source.As a result there is a growing interest in converting bio-waste into bio hydrogen as a source of alternative energy. However the bio-waste are not easily amenable to the fermentation for bio hydrogen production because of the presence of complex carbon sources like carbohydrate, fats and proteins. The micro organisms and their enzymes have the potential to degrade these complex carbon sources and ferment them to different simple substrates which can be utilised for energy generation in the form of bio gases (H_2 and CH_4) ethanol. In the present study we are looking for fermentative bacteria producing hydrolysing enzymes as well as having efficient hydrogen production ability so that single or consortium of microbes can directly convert complex biomass in to biogas (H_2).

Achievable targets

- Collection of environmental sample from different parts of North East India.
- Isolation and screening of fermentative hydrogen producers.
- Checking production of hydrolyzing enzymes by the isolated bacterial species.
- Identification of potential hydrogen producing bacterial strains.
- Optimization of their growth condition and assaying the bacterial strains.

- Utilization of cellulosic waste for hydrogen production.
- Increasing the efficiency of production by various approaches.

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Objective 1: Isolation, preliminary screening for H_2production, extracellular enzyme production and Identification of bacterial strains.

Experimental Methods:

Sample collection

During the project duration different environmental samples were collected from different parts of Assam and Arunachal Pradesh. From each of these locations, samples were collected from the upper surface and from 10—15 cm below the surface in small pre-labelled sterile plastic containers, which were tightly sealed and transported to the laboratory.

Isolation of bacterial strains

To isolate the bacterial strains, 1 g of each sample were suspended in 9ml of sterile 0.85% normal saline solution by vortexing for 2 min on maximum speed. Following a serial dilution of the suspension was made in 0.85% normal saline solution. Thereafter 100ul of each dilution in the series was spread onto the surface of Nutrient Agar media and MSM agar media (with different carbon sources) using the standard spread plate technique. All plates were then incubated at 37^0C for 24-48 hours. From the growth observed over 24-48 hours, various colonies were selected based on their morphology, size and colour. The colonies selected were then streaked out on separate Nutrient Agar media and MSM agar media (with different carbon sources) plates to ensure purity. Colonies were further sub-cultured on above solid media plates if more purification was required. After purification, the cultures were compared visually to eliminate those of similar size, morphology and colour.

Screening for Biogas hydrogen producer

Incubation assembly setup:

For this, 300ml or 125ml anaerobic fermentation assembly were used. The assemblies were made anaerobic by flushing N_2 for 10 minutes. Biogas hydrogen was collected by water displacement method under acidic water.

Experimental condition for Biogas hydrogen production:

Experimental conditions were standardized for biogas hydrogen production as follows-

i) Glucose solution (2%): 250mlof glucose solution was prepared in MSM broth and inoculated with the bacterial isolates.

ii) Temperature: Incubation temperature was 37^0C.

iii) Incubation time: Incubation was carried out till the gas production seased.

iv) pH : Incubation pH was set to neutral.

Checking production of extracellular hydrolyzing enzymes

The productions of extracellular hydrolyzing enzymes were checked by well diffusion method. For this the bacterial strains were grown on Broth medium. Circular wells of 6mm were punched in to the agar plates containing different carbon sources. 100ul of the overnight broth culture and 100ul of the cell free supernatant (centrifuge at 10,000rpm for 10 minute) was then dispensed in each well and incubated for overnight. The zone of hydrolysis by cellulase and amylase was visualised by staining with Grams iodine solution. The protease and lipase activity were measured by visualising clear zone by well diffusion method.

Molecular Identification of the potential hydrogen producers

For molecular characterization 16S rRNA gene sequencing was done using the universal primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′GGTTACCTTGTTACGACTT-3′). The PCR amplifications were performed in Eppendorf

thermocycler in a total volume of 25 µl reaction mixture containing 5 µl of 10X Taq Buffer containing $MgCl_2$, 14.8 µl nuclease free water, 2 µl dNTPs,1 µl of each primers, 0.2 µl of Taq DNA polymerase, and 1 µl of DNA template for each strain. Amplification parameters consisted of an initial denaturation step at 95 °C for 5 min, followed by 30 cycles of primer annealing for 30 sec at 53 °C, elongation for 1 min at 72 °C, and finally 10 min extension at 72 °C. PCR products were separated by electrophoresis in 1% (w/v) agarose gel. The PCR products were purified and used for the automated DNA sequencing using 3130 Genetic Analyzer (Applied Biosystem, Switzerland). The obtained sequences were subjected to NCBI (National Centre of Biotechnology information) BLAST search tool in order to retrieve the homologous sequences in Genbank.

Analytical Methods:

The amount of biogas evolved during fermentation was measured by water displacement method. The gas components were analyzed by gas chromatograph (Nucon GC5765, India) equipped with Porapak-Q and molecular sieve columns using thermal conductivity detector. Argon was used as carrier gas with flow rate 20mL min^{-1} and oven, injector, detector temperature were set to 60, 80 and 110 °C respectively. The glucose concentration was measured by DNS method.

Results:

Isolation and Screening of H_2 producer

Out of 157 isolated strains screened for Biogas hydrogen production 10 strains were found to efficient producer of hydrogen gas, viz. DH1, DH2, DH6, DH13, DH49, DH79, DH89, B1, B6, S3 were taken for further study.

Table: 1- Selected High H_2 yielding bacterial strains:

S. No.	Strain No.	Days after incubation	H_2 Volume (ml)	H_2 content (%)	Yield (Lt H_2/mol glucose)
1	DH1	6	660	61	23.8
2	DH2	6	780	63	28
3	DH6	6	540	48	19.4
4	DH13	6	620	59	22.32
5	DH 49	6	540	50	19.4
6	DH 79	6	510	59	18.4
7	DH89	6	980	65	35.3
8	S3	6	465	67	16.74
9	B1	6	450	48	16.2
10	B6	6	730	65	26.3

Evolution pattern of Biogas and hydrogen by high yielding strain DH89

The production pattern was studied with the high yielding strain and it was observed that the gas production stars from day 2 onwards till day 5 and then a sudden decrease in day 6 due to substrate exhaustion. The hydrogen production was observed in a range between 150-300 ml per day.

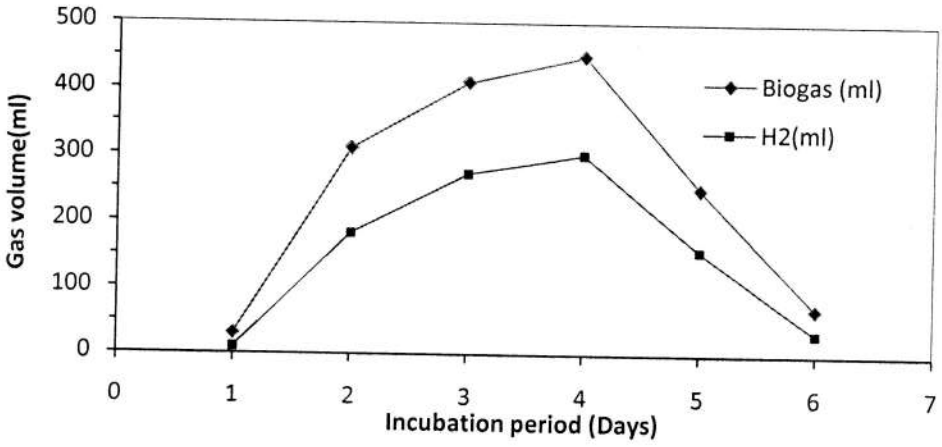
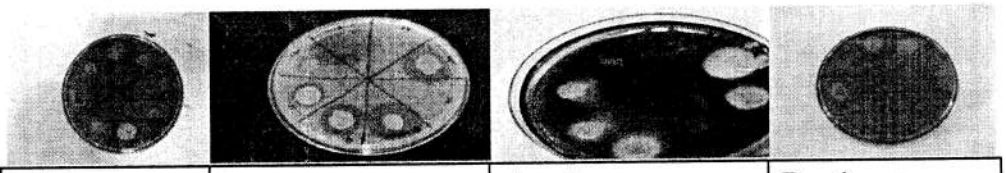


Fig: 1 Evolution of biogas pattern by high yielding strain DH89.

Screening for extracellular enzymes:

Out of 157 strains screened, 19 strains were found to produce little or more all the screened enzymes.Out of 19 strains, 11 strains were found extracellular cellulase positive. Thus, these strains can be used in hydrolysis process of cellulosic waste materials.

Table 2: Selected bacterial strain producing extracellular enzymes



Strain	Cellulase	Protease	Amylase	Pectinase
DH1	+++	-		+++
DH2	+++	-	++	++
DH3	++	++	-	+++
DH5	-	-	+	-
DH6	+	+	+	++
S3	+++	+	+	++
RDB4	+++	+	++	+
DH8	+ +	++	+	-
DH9	-	+	++	-
DH13	+	+++	-	-
DH15	-	-	+	+
DH17	++	-	++	-
DH18	+	++	+	-
DH19	-	+	+	-
DH20	-	+	-	+
DH21	-	+	-	+
DH79	++	++	-	-

DH23	-	++	-	-
DH89	+++	++	-	-

- (no activity), + (very less), ++ (moderate), +++ (high)

Molecular Identification:

The sequenced16S rRNA gene of the selected high H_2 yielding strain was aligned with gene bank NCBI (http://blast.ncbi.nlm.nih.gov) using BLAST program.The gene sequence was also submitted to the NCBI gene bank with a gene bank accession number. The isolated strains are mainly *Bacillus*, one species of *Enterobacter, Staphylococcu*and *Burkholderia*was identified.

Strain name	**Identified as**(Accession No)
DH1	*Penibacillus* sp.(KP723363)
S3	*Bacillus amyloliquefaciens*(KF113840)
DH2	*Burkholderiasp*(KJ867172)
DH89	*Enterobacter cloacae*(KP723362)
B1	*Bacillus sp.* (KT072717)
B6	*Staphylococcus epidermidis*(KT072716)
DH6	*Bacillus* sp.
DH49	*Bacillus* sp.
DH79	*Bacillus circulans.*
DH 13	*Bacillus* sp.

Objective 2: Optimization of culture parameters.

Experimental procedure:

The parameters crucial for fermentative H_2 production including initial pH, and substrate concentration were optimised under batch culture condition. The effect of pH was studied by adjusting the initial pH of the fermentation medium with the pH range of 5 – 9 with incremental step of 1. The fermentation was carried out at 37 °C with carbon source concentration 20 g/L. The effect of glucose concentration on fermentation was studied by varying the initial load from 10 – 50 g/L under optimum pH level at 37 °C.

H_2 production with different carbon source

The ability of the selected strains for utilisation of other carbon sources was studied at optimum pH and incubation temperature 37 °C. The different carbon sources used were glucose, fructose, sucrose, mannose, lactose (10 g/L) and glycerol (10 mL/L).

Analytical Methods:

The amount of biogas evolved during fermentation was measured by water displacement method. The gas components were analyzed by gas chromatograph (Nucon GC5765, India) equipped with Porapak-Q and molecular sieve columns using thermal conductivity detector. Argon was used as carrier gas with flow rate 20mL min^{-1} and oven, injector, detector temperature were set to 60, 80 and 110 °C respectively. The glucose concentration was measured by DNS method.

Results:

Effect of initial pH on H_2 production glucose utilisation and cell growth:

The effect of initial pH on H_2 production by the isolated strains was investigated by varying the initial pH 5 to 9 (with an interval of pH 1) of the fermentation medium. The results showed that the initial pH of the medium is an important factor in the

bacterial growth and H_2production process. The strains were found to produce hydrogen in a wide range of pH though the maximum production was mainly in pH 6, 7 or 8. pH ranging bellow that and above were found not suitable for bacterial growth and as a result low hydrogen yield. The stain viz. DH 6, DH13 and S3 were found to produce maximum hydrogen at media pH 6, strains DH1, DH49, DH89, B1 and B6 were found to produce maximum hydrogen at neutral pH and in case of DH2 and DH79 the production was maximum at pH 8.

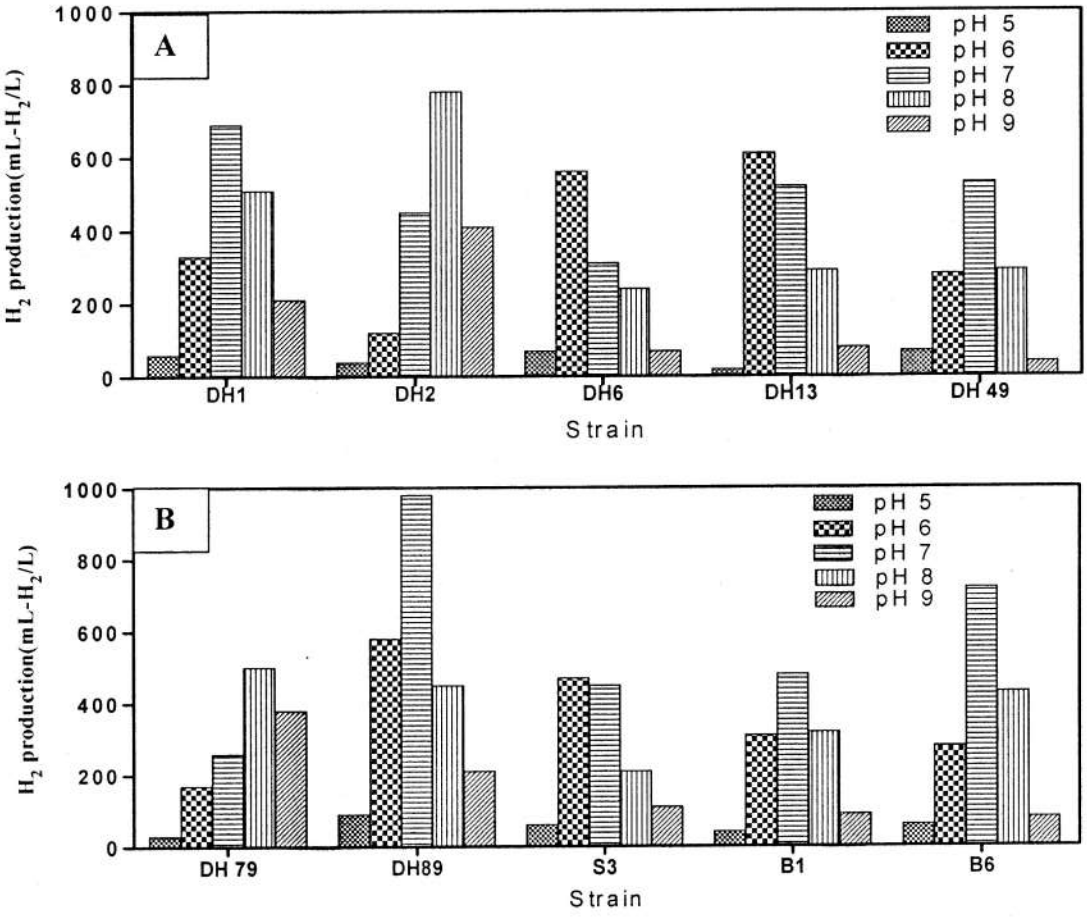


Fig 2 (A & B):- Effect of media pH on hydrogen production.

Effects of initial glucose concentration:

The initial substrate load usually plays a crucial role in cell growth and H_2 production. The results showed that the concentration of 20 g/L is best suitable for maximum

hydrogen yield. By increasing the concentration above this the rate of hydrogen production and hydrogen yield decrease gradually.

Table 3: Effects of initial glucose concentration on H_2 yield, cell growth and glucose consumption efficiency.

Strain	Initial glucose (g/L)	H_2 (ml)	H_2Yield[a]	Cell growth[b]	Final pH	% glucose consumed
DH1	10	210	15.12	0.75	6.2	99.7
	20	680	24.5	1.2	5.7	99.0
	30	610	14.6	1.1	6.1	89.0
	40	640	11.52	1.0	6.3	76.9
	50	690	9.9	1.0	6.7	62.0
DH2	10	320	23.0	0.69	5.9	98.9
	20	810	29.16	1.25	5.7	99.0
	30	850	20.34	0.97	6.1	88.3
	40	900	16.2	0.85	5.8	71.4
	50	970	14	0.83	5.9	65.3
DH6	10	210	15.12	0.92	6.1	99.2
	20	590	21.24	1.45	5.4	99.1
	30	640	15.36	1.4	6.3	84.2
	40	780	14	1.2	5.9	77.9
	50	760	11	1.3	5.8	58.0
DH13	10	270	19.44	0.73	5.7	99.3
	20	690	24.84	0.98	5.6	99.7
	30	620	14.88	1.15	6.3	79.7
	40	510	9.1	1.02	6.1	63.4
	50	590	8.5	1.1	6.1	51.0
DH49	10	210	15.12	0.65	5.3	99.0
	20	590	21.24	0.95	5.6	99.6
	30	600	14.4	0.87	5.4	82.0
	40	540	9.72	0.88	5.9	72.7
	50	400	5.76	0.91	6.3	57.5
DH79	10	290	20.88	0.73	5.1	99.6
	20	610	21.96	0.87	6.2	99.8
	30	640	15.36	0.92	6.6	79.6
	40	510	9.18	0.96	5.9	71.2
	50	590	8.49	0.98	6.5	59.0
DH89	10	470	33.84	0.91	5.3	99.8
	20	990	35.64	1.32	5.6	99.9
	30	830	19.9	1.13	6.1	84.2
	40	850	15.30	0.94	6.6	71.8
	50	890	12.81	0.81	5.9	63.5

S3	10	210	15.12	0.69	5.7	99.9
	20	490	17.64	0.97	5.1	99.8
	30	590	14.16	0.63	5.9	73.0
	40	570	10.26	0.66	6.2	71.0
	50	510	7.34	0.72	6.1	54.8
B1	10	210	15.12	0.54	5.9	99.2
	20	490	17.64	0.78	5.3	99.8
	30	590	14.22	0.64	5.7	81.5
	40	430	7.7	0.69	6.3	77.9
	50	480	6.91	0.48	6.3	63.2
B6	10	320	23.04	0.63	5.2	98.9
	20	760	27.36	0.91	5.3	99.6
	30	810	19.44	0.95	5.4	79.7
	40	840	15.12	0.87	5.7	69.3
	50	730	10.51	0.81	6.3	57.7

[a](Lt H_2/mol glucose fed), [b]Dry cell weight (g/L)

Hydrogen production with different carbon sources:

It is important for H_2 producing bacterial strain to have the ability to use various carbon sources for better utilisation of complex waste biomass. A variety of carbon sources have been reported for fermentative H_2 production. Therefore, different carbon sources were fed at a initial concentration of 10g/L under batch culture condition for evaluate their effect on H_2 production by the isolated strains. The hydrogen production data (Table 4) showed that the isolated strains can utilise diverse carbon sources.

Table 4: Production of H_2 from different carbon sources by the isolated strains

Strain	**Carbon source (10g/L) and hydrogen yield (ml/g carbon source)**						
	Xylose	**Glucose**	**Maltose**	**Fructose**	**Sucrose**	**Lactose**	**Glycerol**
DH1	390	320	280	170	220	170	110
DH2	410	360	170	120	210	310	130
DH6	130	270	180	190	230	140	90
DH13	210	295	280	170	190	110	70
DH49	120	260	310	260	280	330	80
DH79	110	240	190	270	210	250	130
DH89	240	470	320	250	340	210	120

S3	90	240	110	140	220	260	50
B1	140	210	220	230	310	270	70
B6	440	340	280	170	210	305	120

Objective 3: Utilization of cellulosic waste for hydrogen production.

Experimental procedure:

Production of endoglucanase:

The strains were grown on Mineral Salt Medium (MSM) spiked with CMC-Na as the sole carbon source. . The MSM medium consisted of macro nutrients (gl^{-1}): $(NH_4)_2SO_4$, 2 ; Na_2HPO_4, 2.61; KH_2PO_4, 4.75; $MgSO_4$, 0.8; Carbon source, 10; Agar, 15; and micronutrients (μgl^{-1}): $CaCl_2$, 500; $MnSO_4$, 100; H_3BO_3, 10; $ZnSO_4$, 70; $FeSO_4$, 1000; and MoO_3, 50. The pH of the medium was adjusted to 7.0 before autoclaving. The 5 ml of inoculum was transferred to 95ml sterile MSM medium and incubated at 37^0C with a shaking speed of 180rpm. After 48h incubation, culture broth was centrifuged (12,000rpm for 10minutes) at 4 ^{0}C to remove the cell and supernatant was used for assay.

Enzyme assay:

The CMCase activity was measured by estimating the reducing sugars liberated from CMC. The reaction mixture contained 0.49ml buffer pH 6.8, 0.1ml enzyme and 0.5ml 1.0% CMC(w/v) prepared in 50mM phosphate buffer pH 6.8. The mixture was incubated at 50^0C for 20 minutes. Reducing sugars formed were estimated by the method of Nelson and Somogyi. One unit of CMCase activity was defined as the amount of enzyme which liberates 1ug of reducing sugar as glucose per minute under assay conditions. Protein concentration was determined by the Lowery's method using bovine serum albumin as a standard.

Hydrolysis of the Sugarcane bagasse and hydrogen production

Sugarcane bagasse was collected from local farmer and washed with water five times and allowed to sun dry. The dried bagasses were then grounded to produce fine powder for pre hydrolysis in different concentration 1,2,3,5,7 percent using distilled water in 250 ml volume inoculated with the S3 strain and incubated at 370C for 3 days. After hydrolysis the microbes other debris were removed by centrifuging at 700rpm for 10 minutes and the hydrolysate obtained is used for gas production studies. The hydrolysate obtained is then diluted with MSM medium in 1:1 ratio and gas production was carried out in batch culture.

Acid hydrolysis of rice straw:

Acid hydrolysis of the hemicellulose fraction of the rice straw was conducted by treating the dry rice straw (1%, 2%, 3%, 5% and 7%, w/v) with diluted H_2SO_4 (0.5 %, v/v) at 121 °C for one hour in autoclave. After hydrolysis, the hydrolysate was filtered through a thin cloth to remove the solid fraction. Overliming of the hydrolysate was done by adding $Ca(OH)_2$ with frequent stirring and adjusting the final pH to 10. The resulting precipitate was removed by centrifugation at 1500 rpm for 15 minutes. The supernatant was then re-acidified by lowering the pH to 7 and again centrifuged. The final supernatant thus obtained was then used for fermentative H_2 production.

Results:

Endo-1,4 -β-D glucanase activity assay and sugarcane bagasse hydrolysis:

The isolated strains were found to produce measurable CMCase after 48 hours of broth cultivation at 37^0C. The activity was found maximum for S3 strain (52 U/ml). Thus cellulosic hydrolysate was prepared by incubating the S3 strain and the grinded sugarcane bagasse for enzymatic hydrolysis and sugar release.

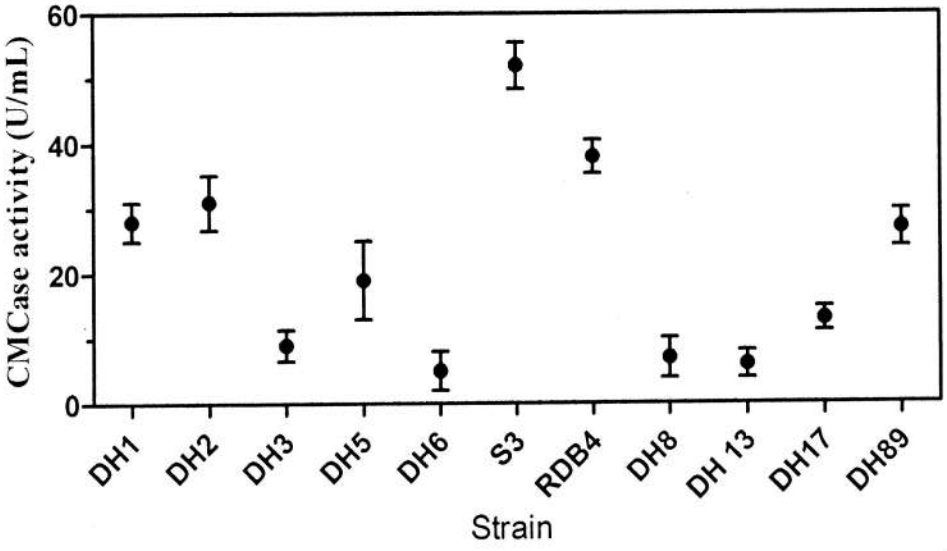


Fig 3: CMCase activity of the isolated strains showing cellulase positive result.

Effect of different substrate on Cellulase production by S3 strain:

The strain S3 was also found to be grow and utilise different low cost bio waste for the better production of cellulase. The production was found maximum when using untreated rice straw (10g/Lt) as substrate with maximum extracellular protein concentration as compare to the other substrates.

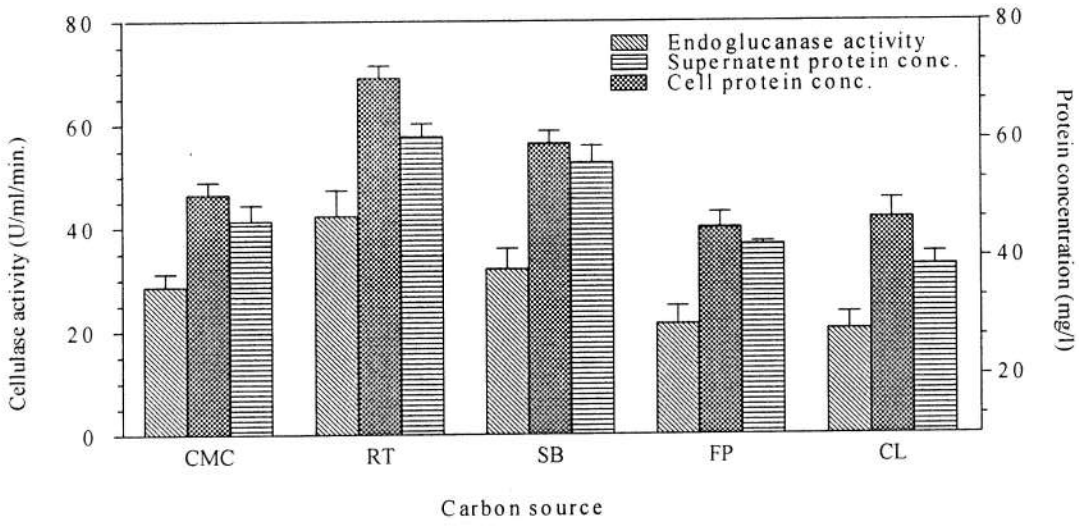


Fig 4: Production of cellulase by S3 strain utilizing different carbon source (CMC, carboxy methyl cellulose sodium salt; RT, rice straw; SB, sugarcane bagasse; FP, filter paper and CL, cellulose).

Hydrogen production from sugarcane bagasse hydrolysate by the strain S3 and DH89:

The high H_2yielding strain S3 and DH89 were tested for hydrogen production from the hydrolysate prepared from sugarcane bagasse. The hydrolysate was prepared by incubating S3 strain a potential cellulase producer, with different concentrations of bagasse and then the sugar released was used for dark fermentation. The hydrolysate was also supplemented with mineral solution for micronutrient source. In both the cases of bacterial fermentation the yield was observed maximum in case of 1 to 3 % feed and then by further increasing the feed concentration results into decrease in yield. In comparison the strain DH89 was found better to utilise the sugarcane bagasse hydrolysate with maximum yield of23.4 L/Kg of the bagasse.

Table 5: Production of H_2 from sugarcane bagasse by S3 and DH89 strain

Fed % (W/V)	(Sugarcane hydrolysate + MSM Media) 9:1					
	Strain					
	S3			**DH89**		
	Biogas Vol(CO_2 + H_2)	H_2 (%)	Yield (L/Kg)	Biogas Vol(CO_2 + H_2)	H_2 (%)	Yield (L/Kg)
1	25	50	12.5	40	51	20.4
2	55	41	11.25	85	55	23.4
3	65	54	11.7	110	56	20.5
5	55	50	5.5	125	55	13.75
7	50	31	2.2	145	46	9.52

Production of H_2 from rice straw hydrolysate by strain B6

The lignocellulosichydrolysate obtained from wood, agricultural waste by-product and crop contains a major fraction of xylose. As in the earlier studies of different sugar utilisation test the strain B6 was found potential in utilising xylose for H_2 production, thus its

feasibility to produce H_2 from acid hydrolysate of rice straw was examined. For this, the different concentration of dried rice straw was treated with diluted H_2SO_4 and the hydrolysate was used for batch fermentation. The maximum H_2 yield of 30 L/kg rice straw was observed with hydrolysate prepared by treating 1% (w/v) rice straw and the yield was decreased by further increasing the rice straw concentration. Diluted acid was used for the hydrolysis process (0.5% v/v). Increasing the acid concentration in acid hydrolysis could provide a strong or complete reaction for hydrolysis, yielding more hydrolyzed product. However, at higher acid concentration the conversion of sugars to various inhibitory compounds takes place, which retard the cell growth. Furfural is one such compound, which is generated as a degradation product from xylose at higher H_2SO_4 concentration. The results suggest that the strain can be used for large scale H_2 production and can help on the way of complete utilization of lignocellulosic waste hydrolysate.

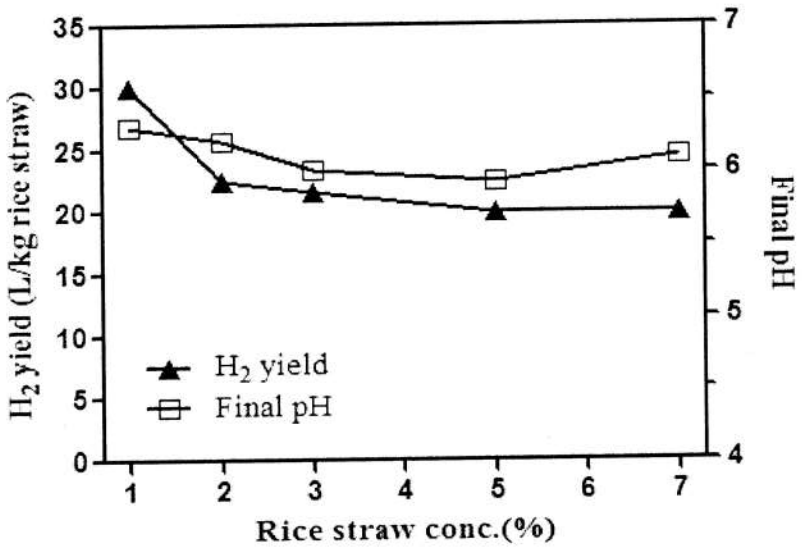


Fig 5: Yield of H_2 and final pH value at different concentration of rice straw hydrolysate by *Staphylococcus epidermidis* B-6.

Objective 3: Increase the production of hydrogen by nanoparticle approach

The synthesis of iron naoparticle and its characterization and application in fermentation media as supplement in place of iron (salt) and studying its effects on H_2 production by isolated strain *E. cloacae* DH 89**:**

Preparation of leaf extract

Freshly collected *S. cumini* leaves were washed three times with water and air dried. The dried leaves were then grinded into fine powder. The extract was prepared by mixing 50 grams of finely powdered leaf with 1 L distilled water, followed by boiling for 10 minutes in boiling water bath. The extract was then filtered through Whatman no.1 filter paper and the filtrate was used as reducing agent for nanoparticle synthesis.

Synthesis of Iron nanoparticles (FeNPs) using leaf extract

Different concentration of leaf extract and FeSO4 solution was reacted to obtain the optimum concentration for synthesis of FeNPs. In a typical synthesis, 25 mL of plant extract was slowly added to 475 mL of 1mM FeSO4 solution with constant stirring for 10 minutes at room temperature. A visible colour change of FeSO4 from light yellow to dark black indicates the formation of FeNPs. The colloidal mixture was then centrifuged (12,000 rpm, 20 °C) for 10 minutes. The pellet was washed with distilled water and freeze dried for further use.

Characterization of the synthesized FeNPs

UV-Visible spectrophotometer (Thermo Fischer Scientific, Evolution 201) was used to perform optical measurements. The UV-Vis spectrum of FeNPs was taken in quartz cuvettes, using distilled water as a reference solvent. UV–Visible analysis was done by continuousscanning from 200 to 600 nm. X–ray Diffraction patterns of the FeNPs was obtained by X– ray diffractometer (Miniflex , Rigaku Corporation Japan). The scanning was

done from 10° to 70° 2θ with 2 min $^{-1}$ of scanning rate. For surface morphology study, Scanning Electron Microscopy (SEM - JEOL JSM-6390 LV, Japan) and Transmission Electron Microscopy (TEM – TECNAI G 20 S-TWIN, 200 kV) analysis were done. The elemental composition of the synthesized FeNPs was determined using EDX spectrophotometer (JEOL-JSM 6390, Japan). FTIR spectra of the aqueous leaf extract and FeNPs was analyzed by FTIR spectrophotometer (Perkin Elmer 1000 FT–IR spectrometer).

Experimental procedure for dark fermentative hydrogen production

Batch experiments were conducted for dark fermentative H_2 production in 125 mL BOD bottle with a working volume of 100 mL under anaerobic condition at 37 °C. The fermentation medium was Mineral Salt Medium (MSM), consisted of macro nutrients (g L^{-1}): $(NH4)_2SO_4$, 2; Na_2HPO_4, 2.61; KH_2PO_4, 4.75; $MgSO_4$, 0.8; glucose, 10; and micronutrients (μg L^{-1}): $CaCl_2$, 500; $MnSO_4$, 100; H_3BO_3, 10; $ZnSO_4$, 70; and MoO_3, 50. The pH of the medium was adjusted to 7.0 before autoclaving. The supplementation of FeSO4 and FeNPs in MSM was done in the range from 0 to 200 mg L^{-1} to evaluate and compare the effect on H_2 production. After bacterial inoculation, the bottles were made air tight using glass stoppers and initial anaerobic condition was established by flushing nitrogen gas. The evolved gases from fermenters were collected by water displacement method under acidic water. The gas collection and analysis was carried out till the gas production was ceased.

Analytical Methods:

The amount of biogas evolved during fermentation was measured by water displacement method. The gas components were analyzed by gas chromatograph (Nucon GC5765, India) equipped with Porapak-Q and molecular sieve columns using thermal conductivity detector. Argon was used as carrier gas with flow rate 20mL min^{-1} and oven,

injector, detector temperature were set to 60, 80 and 110 °C respectively.

Results:

UV–vis spectroscopy and XRD study of synthesised Iron nanoparticles:

The formation of FeNPs was visually observed by change in colour of the parent precursor and reducing agent mixture during the synthesis. Figure 6a shows the UV–vis spectra of the aqueous solution of FeSO4 and the synthesized colloidal material. A prominent difference can be observed in the spectra. The synthesized FeNPs showed absorption from 350 nm with a peak at 267 nm, which is typical for iron nanomaterials. The XRD pattern of the FeNPs (figure 6b) is complex and shows several small intensity diffraction peaks. The prominent diffraction peaks can be indexed to α-FeOOH (JCPDS no. 29-0713), Fe2O3 (JCPDS no. 33-0664) and Fe3O4 (JCPDS no. 19-0629). Most of the peaks from the phases overlap each other. Since the peaks are not intense, it is difficult to determine the crystallite size using the Scherrer formula.

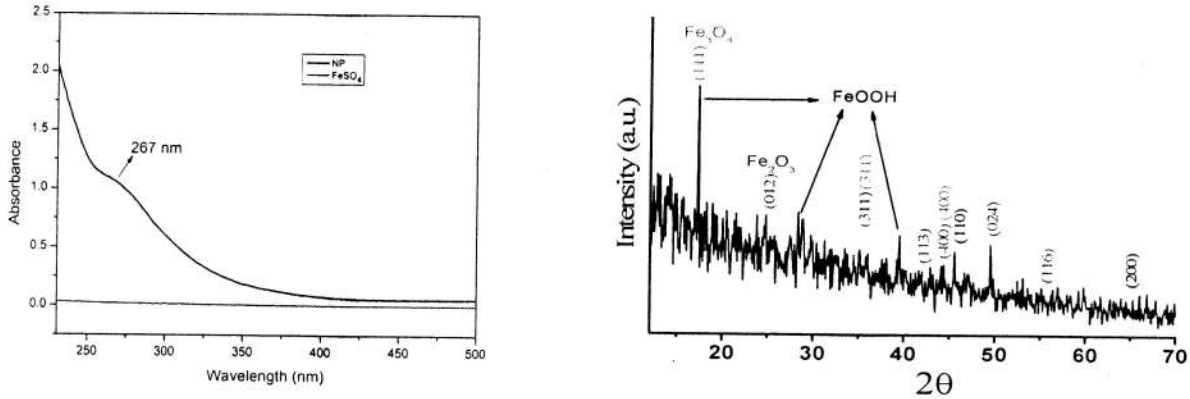


Fig: 6 (a) UV–vis spectra of $FeSO_4$ and synthesized NPs, (b) XRD pattern of NPs.

SEM, TEM, EDX and FTIR analysis:

The morphology of the FeNPs was investigated by electron microscopy. Figure 7A shows the SEM image. It indicated the formation of random shaped aggregates of micron-sized particles. It can be attributed to uncontrolled nanoparticle agglomeration during the synthesis.

This fact is clearly evident from the TEM images (figure 7B). It showed agglomerated clusters of nanoparticles of primary sizes in the range of 20–25 nm. Careful observation of the TEM images indicated that the material is porous. The EDX spectrum (figure 8A) shows the elemental profile of FeNPs, primarily composed of C, O, S and Fe. The C and O are mainly from the compounds present in plant extracts, while Fe and S from the FeSO4 precursor. The elemental percentage was observed as 40.54% C, 50.82% O, 2.45% S and 6.19% Fe. The FTIR spectra of the plant extract (a) and FeNPs (b) are shown in figure 8B. After comparing the spectra, it was observed that some peaks were shifted and missed. However, few additional peaks were also emerged in case of synthesized FeNPs.

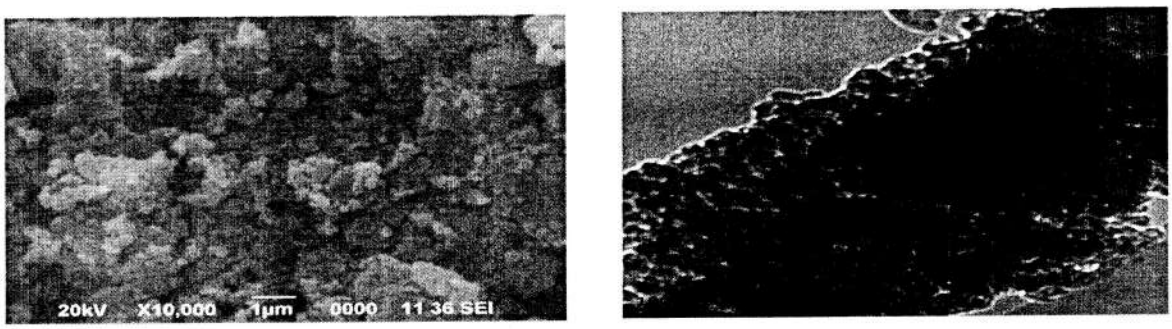


Fig 7: (A) SEM image of FeNPs (B) TEM image of FeNPs

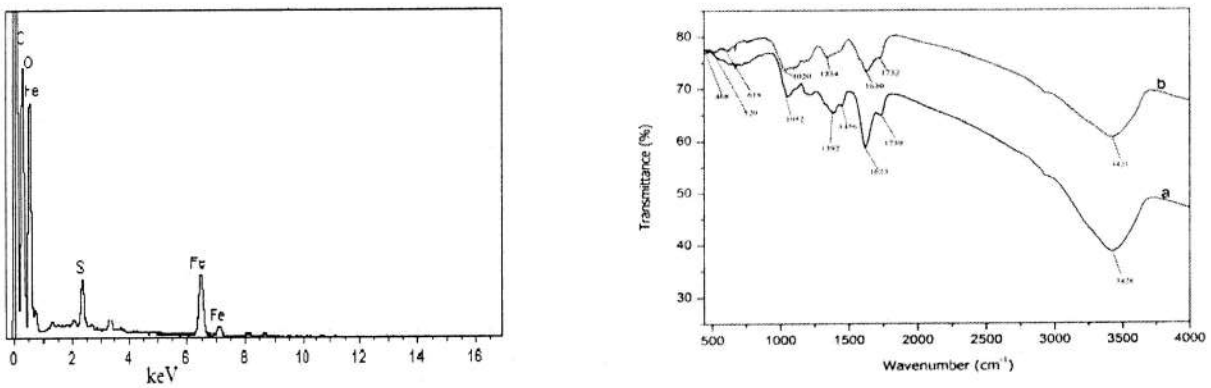
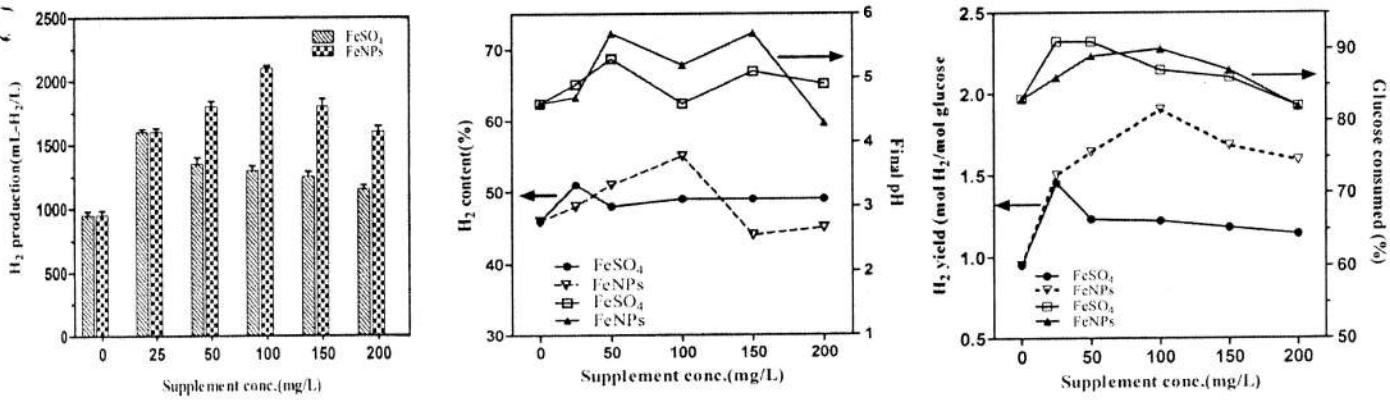


Fig:8 (A) EDX pattern of NPs (B) FTIR of NPs and plant extract

Effects of $FeSO_4$and FeNPs on batch fermentative H_2 production:

The effect of $FeSO_4$ and FeNPs on fermentative H_2 production using *E. cloacae* DH-89 is shown in figure 10. It was found that the H_2 production increases on the addition of both

the form of iron. The H_2 production in the control (no supplementation) was 950 ml/l of fermentation media, which was noticeably increased by supplementation of $FeSO_4$ at a concentration of 25 mg/l and then decreased with further increase in the concentration from 50 to 200 mg/l. With the optimum concentration of 25 mg/l $FeSO_4$ supplementation the H_2 production was increased to 1600 ml/l. The addition of high concentration of $FeSO_4$ (above 25 mg/l) suppressed the activity of bacterium, consequently a low H_2 production. A similar kind of result, i.e., the higher concentration of $FeSO_4$ addition decreases the H_2 production has been reported. However, supplementation of green synthesized FeNPs in place of $FeSO_4$ was found to further increase the H_2 production. The maximum H_2 production of 2100 ml was achieved at 100 mg/l FeNPs supplementation and it was decreased by increasing the concentration above 100 mg/l. The present finding suggests that the supplementation of iron can be replaced by ironbased nanoparticles for better H_2 production. The fact is that, iron is a fundamental component of ferredoxin, which acts as an electron carrier in the hydrogenases, responsible for H_2 production. Hence, at a certain concentration of iron or FeNPs supplementation increases the ferredoxin activity during the fermentation process, which leads to enhanced H_2 production.



(a) (b) (c)

Fig: 9 (a) H_2 production at different concentrations of $FeSO_4$ and FeNPs supplementation. (b, c) Effects of $FeSO_4$ and FeNPs supplementation on H_2 yield and glucose consumption, H_2 content and final pH.

Dark fermentative hydrogen production by the high xylose utilising isolate *Staphylococcus epidermidis* B-6 from xylose:

Xylose shares a major fraction (35–45%) of total sugar yield from hydrolysis of lignocellulosic materials. Extensive studies have been done on fermentative H_2 production from glucose and sucrose. However, due to inefficiency of microbes for xylose utilization, there are a very few reports on H_2 production from xylose using pure culture of microbes. Hence, for complete utilization of sugar released from lignocellulosic material it is important to isolate bacterial species with efficiency to convert xylose to H_2.

Experimental methods:

Optimization of culture condition:

The parameters crucial for fermentative H_2 production including initial pH, nitrogen source and substrate concentration were optimised under batch culture condition. The effect of pH was studied by adjusting the initial pH of the fermentation medium with the pH range of 5 – 9 with incremental step of 1. The fermentation was carried out at 37 °C with xylose concentration 10 g/L. The effect of nitrogen source on H_2 production was studied by fermentation medium GM-2 amended with inorganic (ammonium sulphate, ammonium chloride) and organic (yeast extract, peptone) nitrogen sources at a concentration of 1.0 g/L. The initial pH was 7.0, fermentation temperature 37 °C and xylose concentration 10 g/L. The effect of xylose concentration on fermentation was studied by varying the initial xylose load from 5 – 50 g/L under optimum pH, and nitrogen source at 37 °C.

Analytical Methods

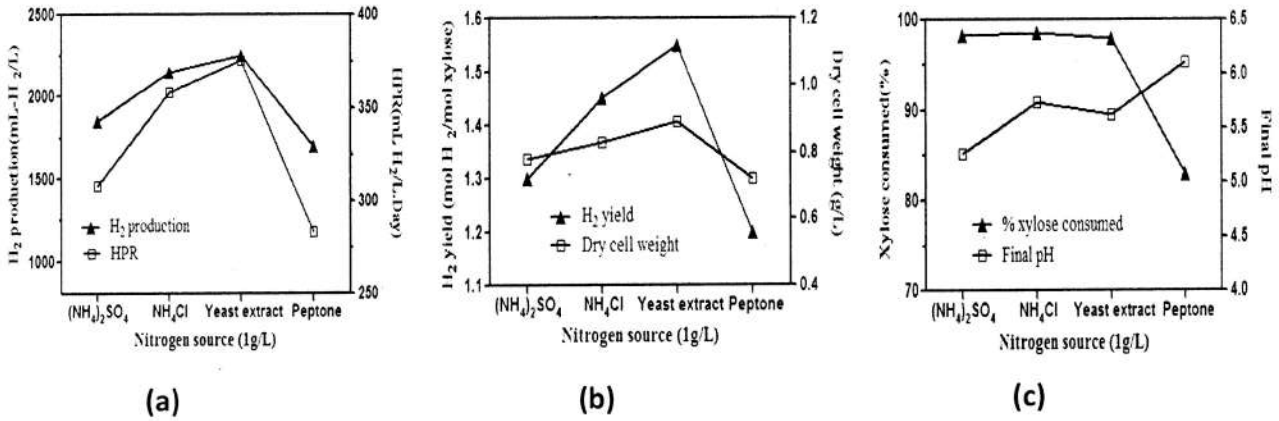
The amount of biogas evolved during fermentation was measured by water displacement method. The gas components were analyzed by gas chromatograph (Nucon GC5765, India) equipped with Porapak-Q and molecular sieve columns using thermal

conductivity detector [12]. Argon was used as carrier gas with flow rate of 20 mL/min and oven, injector, detector temperature were set to 60, 80 and 110 °C respectively. The xylose concentration was measured by DNS method [13].

Results:

Effect of various nitrogen sources on H_2 production

The effect of various nitrogen sources on H_2 production by *Staphylococcus epidermidis* B-6 is shown in Fig. 10(a–c). A significant change in H_2 production was observed by changing the source of nitrogen in the fermentation medium. The maximum H_2 yield of 1.55 mol H_2/ mol xylose was observed using yeast extract with highest bacterial growth and 98% of xylose consumption (Fig. 10 a,b). This is due to the fact that yeast extract is a complex nitrogen source comprising of peptides and amino acids, which can be easily taken up by the bacterium during fermentation and directly incorporated into proteins or transformed into other cellular nitrogenous constituents. On the other hand, when inorganic nitrogen sources are used, the cells has to spend more energy in synthesizing amino acids as a result they spend a longer period of lag phase and decrease in H_2 yield. The results suggest that yeast extract as better nitrogen source for maximum H_2 production with high production rate and bacterial growth. However the other cheap nitrogen sources like ammonium chloride and ammonium sulphate also showed relatively higher production of H_2 (Fig.10 a). Thus, this can be beneficial for industrial scale H_2 production by using cheap inorganic nitrogen sources instead of expensive organic nitrogen sources.



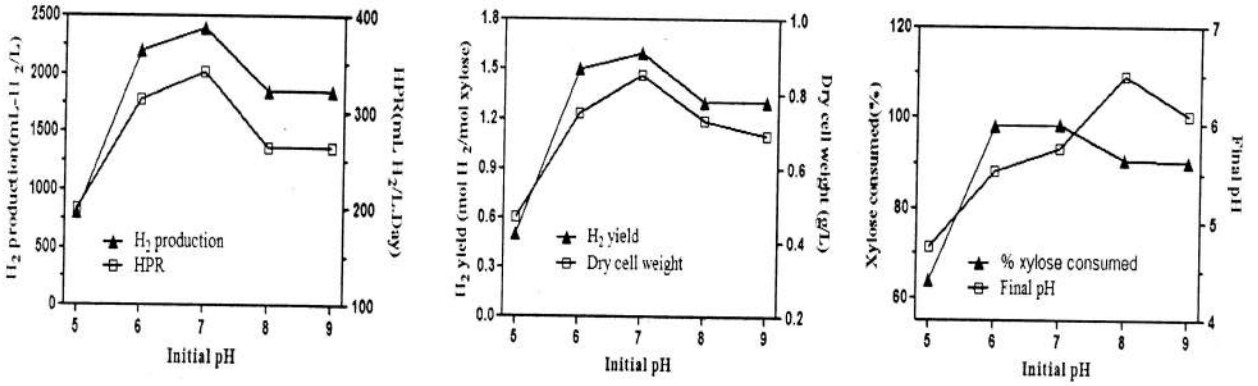
(a) (b) (c)

Fig. 10 a-c The effect of N_2 source on H_2 production performance of *Staphylococcus epidermidis* B-6. **a** Volume and rate of H_2 production. **b** H_2 yield and bacterial growth. **c** Xylose degradation rate and final pH value.

Effect of initial pH on H_2 production

The effect of initial pH on H_2 production by *Staphylococcus epidermidis* B-6 was investigated at pH 5 to 9 (with an interval of pH 1). The result (Fig.11 a–c) showed that the initial pH of the medium is an important factor in the bacterial growth and H_2 production process. The yield of H_2 and cell growth was increased significantly by increasing the initial pH from 5 to 7 and then decreased by further increasing the pH (Fig 11b). The rate of hydrogen production (Fig 11a) and xylose degradation (Fig 3c) also showed a similar trend with cell growth and H_2 yield as shown in Fig. 11b. The maximum H_2 yield of 1.6 mol H_2/mol xylose with hydrogen production rate of 342 mL H_2/L.Day was observed at pH 7. The cell growth and xylose consumption was also observed maximum at this pH. The H_2 yield and cell growth was very low at pH 5 and bellow that no growth and H_2 evolution was observed. This can be the fact that, at high concentration of H^+ ion environment, the cell's ability to maintain internal pH get destabilised, consequently intracellular ATP level drops and inhibiting xylose uptake. However, the strain B-6 was found to produce H_2 above 1 mol/mol xylose within the pH range of 6 to 9 and very little or no gas production was observed by further increasing the pH. This can be the fact that at higher pH range the

activity of the key enzyme [Fe – Fe] hydrogenase get decreased and the changing direction of metabolic pathway from acidogenesis to solventogenesis results into low H_2 production



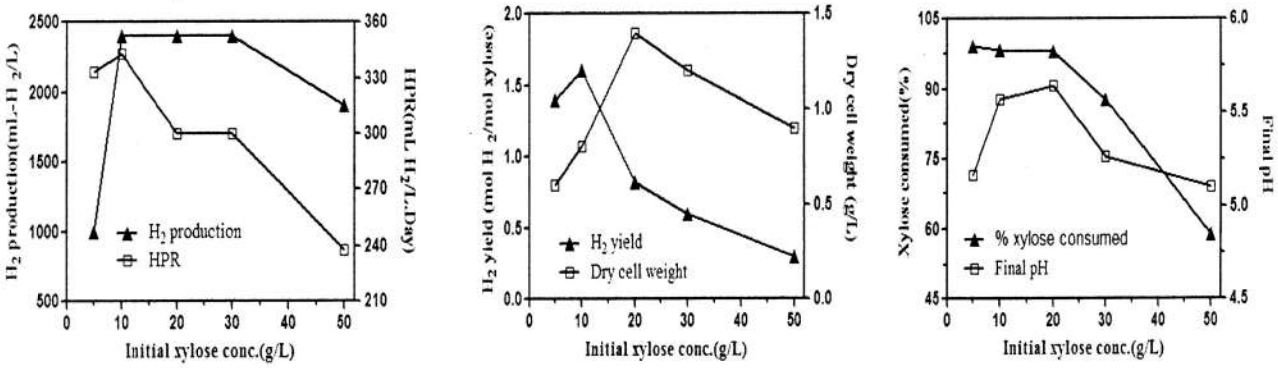
(a) (b) (c)

Fig. 11 a-c The effect of initial pH on H_2 production performance of *Staphylococcus epidermidis* B-6. **a** Volume and rate of H_2 production. **b** H_2 yield and bacterial growth. **c** Xylose degradation rate and final pH value.

Effect of initial xylose concentration on H_2 production

The initial substrate load usually plays a crucial role in cell growth and H_2 production. Fig 12(a–c) shows the various effects of initial xylose concentration on fermenatation. An increase in the H_2 yield from 1.4 to 1.6 mol H_2/mol xylose was observed by increasing the xylose concentration from 5 to 10 g/L and was then decreased by further increasing the concentration. Though the volume of H_2 production (Fig 12a) was relatively higher by increasing xylose concentration above 10 g/L, but the rate of production was found gradually decreases with H_2 yield and xylose consumption rate (Fig. 12 b,c). However cell growth was observed maximum at 20 g/L xylose concentrations and was then decreased above this concentration. This is due to the fact that at higher substrate concentration the yield is decreased by inhibitory effect of substrates. Another reason may be that at higher substrate concentration, the carbon flux is directed more towards the production of reduced by-

products like organic acids and alcohols. The un-dissociated organic acids get accumulated in the fermentation broth with higher substrate concentration, which would leak into the cell and decrease the pH of the intracellular environment. As a consequence, the cell growth and H_2 yield get inhibited.



(a) (b) (c)

Fig. 12 a-c The effect of xylose concentration on H_2 production performance of *Staphylococcus epidermidis* B-6. **a** Volume and rate of H_2 production. **b** H_2 yield and bacterial growth. **c** Xylose degradation rate and final pH value.

B2. Summary and Conclusion of the Progress made so far (minimum 100 wards, maximum 200 words)

In the present study successful isolation and identification of 10 mesophilic hydrogen producing bacterial has been done and the optimization for the maximum gas production using glucose as sole carbon source was performed. The conditions crucial for hydrogen production by pure culture of isolates were like pH and initial carbon source load. These two parameters were tested and finally the maximum hydrogen gas producing strains were identified. DH 89, B6, S3, DH1 are some potential strains which can be used for utilisation of various bio-waste. Strain S3 was also found a potential strain for production of extracellular enzyme for degrading of cellulosic material. The ability of the strain practically tested with waste sugarcane baggasse for sugar release and finally hydrogen production. Strain DH 89 and S3 were examined for hydrogen production from sugarcane baggasse hydrolysate. The

hydrogen production was recorded as 23.5 L/Kg and 12.5 L/kg respectively for DH89 and S3 from sugarcane baggasse. The strain B1 was found potential candidate for xylose utilisation and hydrogen production from xylose released from acid treated rice straw was tested and was found 30 L/kg of rice straw. It is important for bacterial strains to utilise mono, di and polysaccharides for growth and hydrogen production. The selected strains were also found potential in utilising of various other sugars for hydrogen production. The major challenge in dark fermentation method of hydrogen production is low yield and slow production rate. Therefore, the search for a novel approach is required to accelerate the H_2 production rate and enhance the yield. In the present study a clean method is applied for the synthesis of FeNPs using water extract of the *S. cumini* leaf. The synthesized FeNPs is used as media supplement in place of iron to enhance the dark fermentative H_2 production by the isolated strain DH-89 in glucose-based batch fermentation. It was found that the yield of hydrogen production increase considerably by replacing iron with iron nanoprticle at a concentration of 100 mg/L. The production was found increased from 1600ml/L to 2400 ml/L. Thus the present study will be great beneficial in the context of eco-friendly and renewable fuel production and reduction of waste generation.

B3. Details of New Leads obtained, if any:- Nil

B4. Details of publications and patents, if any:-1 publication, 1 communicated.

1. Phytosynthesized iron nanoparticles: effects on fermentative hydrogen production by*Enterobacter cloacae*DH-89., *Bull. Mater. Sci.*, Vol. 38, No. 6, October 2015, pp. 1533–1538.

2. Hydrogen production by newly isolated *Staphylococcus epidermidis* B-6 from xylose and rice straw hydrolysate. Communicated in *Current microbiology*, (Manuscript Number: CMIC-D-16-00080, under review)

Future Work:

- The isolated strains can be checked for hydrogen production in continuous culture mode of fermentation.
- The bacterial strains can be immobilised in various materials and reusability of the immobilised strain can be checked.
- Consortium of microbes can be formed with the isolated strains for maximum hydrogen yield as well as waste utilisation.

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Appendix-B

FINAL UTILIZATION CERTIFICATE

(For the entire project period 2011-2016)

1.	**Title of the Project/Scheme**:	"Isolation and characterization of hydrogen Producing bacteria from North-Eastern states of India,(With special emphasis on Assam and Arunachal Pradesh), for efficient conversion of biomass to hydrogen".
2.	**Name of the Organisation:**	Tezpur University, Assam.
3.	**Principal Investigator**:	Dr. Manabendra Mandal.
4.	**DBT sanction order No. & date of sanctioning the project**:	Order No. - BT/212/NE/TBP/2011 dated December 14, 2011
5.	**Amount brought forward from the previous financial year quoting DBT letter No. & date in which the authority to carry forward the said amount was given:**	**NIL**
6.	**Amount received from DBT during the financial year (*please give No. and dates of sanction orders showing the amounts paid)*:**	**(Rs. 21,82,000 + Rs 5,19,000) = Rs. 27,01,000**
7.	**Other receipts/interest earned, if any, on the DBT grants:**	**Rs. 60,252 (Interest earned)**
8.	**Total amount that was available for expenditure during the financial year (Sl. Nos. 5,6 and 7):**	**Rs. 27,61,252**
9.	**Actual expenditure (excluding commitments) Incurred during the financial year (statement of expenditure is enclosed):**	**Rs. 26,22,855**
10.	**Unspent balance refunded, if any *(Please give details of cheque No. etc.):***	**NIL**
11.	**Balance amount available at the end of the financial year:**	**Rs. 1,38,397**

12. **Amount allowed to be carried forward to the next financial year vide letter No. & date:** Nil

1. Certified that the amount of **Rs. 26,22,855.00** mentioned against col. 9 has been utilised on the project/scheme for the purpose for which it was sanctioned and that the balance of **Rs. 1,38,397.00** remaining unutilized at the end of the year has been surrendered to Govt. (vide No. _____ dated _____)/will be adjusted towards the grants-in-aid payable during the next year.

2. Certified that I have satisfied myself that the conditions on which the grants-in-aid was sanctioned have been duly fulfilled/are being fulfilled and that I have exercised the following checks to see that the money was actually utilised for the purpose for which it was sanctioned.

   Kinds of checks exercised:

1.

2.

3.

4.

5.

(PROJECT INVESTIGATOR)

31/2/14

(FINANCE OFFICER)

Finance Officer
Tezpur University

(HEAD OF THE INSTITUTE)

Registrar
Tezpur University

(To be countersigned by the DBT Officer-in-charge)

Appendix- C

Statement of Expenditure referred to in para 9 of the Utilisation Certificate

Showing grants received the Department of Biotechnology and the expenditure incurred **for the entire project period 2011-16**

		Financial Year- wise grant release (Rs)					Total grant release (Amount in Rs)	Financial Year- wise expenditure (Amount in Rs)					Total expendi--ture (Rs)	Balance (Rs)	Interest earned (Rs)	Final Balance at project ending
Heads	Sanctio--ned Amount	(2011-12)	(2012-13)	(2013-14)	(2014-15)	(2015-16)	(B+C+D+E+F) = G	(2011-12)	(2012-13)	(2013-14)	(2014-15)	(2015-16)	(H+I+J+K+L) = M	(G - M)	(N)	(G - M + N)
	A	B	C	D	E	F		H	I	J	K	L				
1. Non-recurring																
(i) Equipments	15,46,000	15,46,000	0	0	0	0		0	11,65,765	3,79,090	00	00				
2. Recurring																
(i)Manpower	6,60,000	2,11,000	0	2,04,000	0	0		0	1,32,000	1,43,999	1,08,000	00				
(ii)Consumables	5,00000	2,00,000	0	1,98,000	0	0		0	1,97,907	00	89,698	1,08,765				
(iii)Travel	1,50,000	50,000	0	29.000	0	0		0	21,310	7,610	9,500	00				
(iv)Contingency	1,75,000	75,000	0	49,000	0	0		0	70,887	3,211	35,323	10,790				
(v)Overheads (if applicable)	2,00,000	1,00,000	0	39,000	0	0		0	89,850	00	60,550	(-) 11,400				
Total	**32,31,000**	**21,82,000**	**00**	**5,19,000**	**00**	**00**	**27,01,000**	**00**	**16,77,719**	**5,33910**	**3,03,071**	**1,08,155**	**26,22,855**	**78,145**	**60,252**	**1,38,397**

(PROJECT INVESTIGATOR)

3/2/16

(FINANCE OFFICER)

Finance Officer
Tezpur University

(HEAD OF THE INSTITUTE)

Registrar
Tezpur University

Annexure A

Manpower Staffing Details **for the entire project period 2011-2016**

(Amount in Rs.)

Name of the person	Name of the post	Date of joining	Date of leaving	Total monthly salary	Total salary paid during project period
Dhrubajyoti Nath	JRF	01/05/2012	1/01/15	12,000	3,83,999

(PROJECT INVESTIGATOR)

3/2/15

(FINANCE OFFICER)

Finance Officer
Tezpur University

(HEAD OF THE INSTITUTE)

Registrar
Tezpur University

Annexure B

Manpower Expenditure Details **for the entire project period 2011-2016.**

(Amount in Rs.)

Sanctioned Posts	Number	Scale of Pay	Annual Outlay	Outlay for The entire Period	Revised Scale, if any	Revised Annual Outlay	Revised Project Outlay	Actual relases by DBT	Actual Expenditure	Balance
JRF	**01**	**12,000 0**	**2,11, 000**	**6,60,000**	**Nil**	**Nil**	**Nil**	**4,15,000**	**3,83,999**	**31,001**

(PROJECT INVESTIGATOR)

3/2/16

(FINANCE OFFICER)

Finance Officer
Tezpur University

(HEAD OF THE INSTITUTE)

Registrar
Tezpur University

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* Details of manpower salary/ fellowship revision along with due- drawn statement and arrears requested should be given separately, if applicable.

Appendix-A

Details of Assets acquired wholly or substantially out of Govt. grants
Register to be maintained by Grantee Institution

	Name of the Sanctioning Authority:	Department of Biotechnology. Government of India.
1.	Sl. No.	92
2.	Name of the Grantee Institution	Tezpur University.
3.	No. & Date of sanction order	Order No. BT/212/NE/TBP/2011 Dated December 14, 2011.
4.	Amount of the sanctioned grant	Rs. 21,82,000 (1st year) & Rs. 5,19,000 (2nd year).
5.	Brief purpose of the grant	To work for the DBT Twinning project entitled "Isolation and characterization of hydrogen producing bacteria from North-Eastern states of India,(With special emphasis on Assam and Arunachal Pradesh), for efficient conversion of biomass to hydrogen".
6.	Whether any condition regarding the right of ownership of Govt. in the Property or other assets acquired out of the grant was incorporated in the grant-in-aid sanction order.	Nil
*7.	Particulars of assets actually credited or acquired.	Attached below.
8.	Value of the assets as on 2014-2015	**Rs. 15,44,855.00**
9.	Purpose for which utilised at present	To work for the DBT–Twinning Project (NE)
10.	Encumbered or not	Nil
11.	Reasons, if encumbered	NA
12.	Disposed of or not	Not
13.	Reasons and authority, if any, for Disposal	NA

14.	Amount realised on disposal	NA
15.	Remarks	Nil

* List of equipment purchased indicating the item wise costs may please be provided.

List of committed Expenditure under non-recurring head:

Sl. No.	Instruments	Status	Cost (in Rupees)
1	Gas Chromatograph	Installed	8,11,463.00
2	Shaking Incubator	Installed	2,64,302.00
3	BOD incubator	Installed	95,340.00
4	Cold centrifuge	Installed	2,83,750.00
5	Weighing balance	Installed	90,000.00
		Total	15,44,855.00

(PROJECT INVESTIGATOR)

31/02/16

(FINANCE OFFICER)

Finance Officer
Tezpur University

(HEAD OF THE INSTITUTE)