Statistical optimization of ethanol production from *Allium ascalonicum* leaves via Consolidated Bioprocessing

*S.Kavitha
Department of Biotechnology
Adhiyamaan College of Engineering
Hosur, Tamil Nadu, India.
kavitha.biotechnology@gmail.com

P.P.Akshai Kumar
Department of Biotechnology
Adhiyamaan College of Engineering
Hosur, Tamil Nadu, India.
akshaikumar7893@gmail.com

K.Jegan
Department of Biotechnology
Adhiyamaan College of Engineering
Hosur, Tamil Nadu, India.
jegankannan312@gmail.com

***Abstract*- The ever-increasing demand for fossil fuel, the limitation of universal petroleum reserves and the impact of greenhouse gases (resulting from petroleum burning) on climatic change have augmented the requirement for sustainable and renewable biological fuels an imperative for the future. Lignocellulosic materials are abundant source for ethanol production. Lignocellulosic biomass constitutes a substantial renewable substrate for bioethanol production that does not compete with food and animal feed and is a promising feedstock for future renewable fuels. Thermophillic bacterium produce cellulase enzyme which hydrolysis pretreated biomass and converts simple sugars to ethanol. The effect of nutrients on cellulase & ethanol yield from pretreated *Allium ascalonicum* leaves using the thermophillic bacterial isolate was estimated and statistically optimized using RResponse Surface Methodology. The present study compares these approaches for cellulase production for enzymatic hydrolysis of *Allium ascalonicum* leaves biomass to maximize cellulosic monomers for ethanol production.**

Keywords: cellulase, consortia, ethanol, optimization, response surface methodology

1. Introduction

Ethanol is a volatile, flammable, colorless liquid with a slight chemical odor and it is a non-toxic fuel. This bio ethanol can be used as a bio fuel in automobiles in order to reduce the amount of carbon emission from the automobiles. Bio ethanol can be produced by converting Lignocellulosic biomass into bio ethanol. (Klein *et al.*, 2016).

Lignocellulosic biomass is a plant dry biomass most abundantly available feedstock for biofuel production, mainly for bioethanol. Lignocellulosic biomass is rich in carbohydrate consists of cellulose, hemicelluloses and lignin. The aromatic lignin which connects the cellulose and hemicelluloses in the plant cell wall. (Klein *et al.,* 2016). The cellulose and hemicelluloses are hydrolyzed by acid pretreatment. (Vandenbossche *et al.,* 2014), (Varma *et al*., 2013). The lignocelluloses feedstock used is *Allium ascalonicum* leaf. This lignocellulosic biomass is converted into bioethanol by consolidated bio processing method using thermophilic cellulolytic bacteria. (Satoshi *et al.,)*.

This bioethanol production is performed by consolidated bio processing at low cost. Consolidated bio processing method follows four biological processes (Production of cellulolytic enzymes, Hydrolysis of Polysaccharides present in Pre-treated biomass, Fermentation of hexose sugar and Fermentation of pentose sugars) in a reactor. The cellulolytic enzyme is produced by the thermophilic cellulolytic bacteria. This bacteria acting as the primary candidate for consolidated bioprocess method. (Satoshi *et al.,)*.

Thermophilic cellulolytic bacteria capable of producing cellulolytic enzyme. Bioconversion of cellulose into bioethanol from *Allium ascalonicum* leaf is performed by cellulolytic bacteria using one factor at a time method. Being cellulolytic and ethanologenic abilities, capable of directly converting a cellulosic substrate into ethanol.

2. Experimental Methods

2.1 Chemicals:

High purity of sodium chloride, carboxy methyl cellulose, dinitro salicylic acid, ammonium chloride, citric acid, disodium hydrogen phosphate, cellulase, sodium chlorite and glucose were procured from Sigma–Aldrich (Bommasandra, Bengaluru, India) used for proximate biochemical analysis. Acetic acid and nitric acid was used for pretreatment of *Allium ascalonicum* leaves was purchased from Merck Chemicals Ltd., Mumbai, India. Additional chemicals and solvents used in this research study purchased from Sigma Aldrich or Merck.

2.2 Sample Collection and Handling:

The *Allium ascalonicum* leaves were collected in polythene bags from the agricultural field of Erode District. The cellulolytic thermophilic microbes were isolated from the compost soil. The *Allium ascalonicum* leaves were dried in hot air oven at 60°C for 3 hrs and grounded to powder. The powdered leaves were then stored in 4°C for further use.

2.3 Proximate Biochemical Characterization:

Moisture, ash, lipid, Starch, protein, cellulose, hollocellulose, hemicellulose and total solids were estimated by standard AOAC 2000 methods.

2.4 Acid Pretreatment of The Sample:

The lignin was removed from the cellulose by acid pretreatment. 1 gram of the sample was taken in triplicate and 3 ml of acetic/nitric reagent was added to each and every test tube and mixed well in a vortex mixer. Then the sample was heated for 30 min in water bath at 100°C. While heating the sample gets changed into orange yellowish in color. The yellow color indicates the presence of lignin content. The residue was washed with distilled water and 1:1 ratio of acetone and ethanol using filter paper until the residue comes to white color. The white color residue indicates the cellulose content in the sample. Initially the filter paper was weighed and the filter paper with the residue was dried in hot air oven for 30 min. The weight of the filter paper with the residue was weighed and the amount of cellulose content was estimated. These pretreated samples were used for the production of bioethanol.

2.5 Isolation of Cellulolytic Bacteria From Compost Soil:

Media has been prepared for isolating cellulolytic thermophilic microbe. The media composition were 1g – peptone, 1g-CMC, 0.2g-K_2HPO_4, 1.5-agar, 0.03g-$MgSO_4.7H_2O$, 0.25g-$(NH_4)_2SO_4$. Compost soil has been taken from our college. 1 gram of the sample was weighed and 10 ml of distilled water was added to it. The sample was then serial diluted up to 10^{-5} dilution rate. The serially diluted sample was then spread plated in the media. From the spread plate a group of colony has isolated and streak plate has done to isolate single colony. The plate was then placed in an incubator for 48 hours at 30°C for them to grow in a suitable condition. Single colony has been isolated from the plate.

2.6 Identification Of Cellulolytic Bacteria:

The microbes were identified morphologically and gram's staining was performed to view the shape and nature of the bacteria.

The CMC agar plates were flooded with 1 % Congo red and allowed to stand for 15 min at room temperature. One molar NaCl was thoroughly used for counterstaining the plates. In other case Gram's iodine flooded over the CMC agar. Clear zones were appeared around growing bacterial colonies indicating cellulose hydrolysis. The bacterial colonies having the largest clear zone were selected for identification and cellulase production in submerged system.

2.7 Cellulase Assay

The culture medium was centrifuged at 5000 rpm for 20 min at 4°C. The supernatant was used as crude enzyme source for cellulase assay. 0.450ml of 1% Carboxy methyl cellulose as the substrates in 0.2M citrate phosphate buffer (pH-5) and incubated at 55°C for 15 min.The reaction was terminated by addition of 0.5ml of Dinitro solicilic acid reagent

and tubes were kept at boiling water bath for 5min. After cooling the tubes at room temperature, 3ml of distilled water was added in each tube. The intensity of the color was read at 540nm.Standard curve was performed with glucose solution. One unit of enzyme activity was defined as the amount of enzyme required for release 1µ mol of glucose per minute under assay condition.

2.8 Biochemical Test:

Biochemical test were performed to identify the type of organism. Indole test, MR-VP test, carbohydrate, citrate test, TSI test were some of the biochemical test performed.

2.9 One-factor-at-a-time Method:

In this one factor at a time method the effective results were observed for all single media components. The media was prepared with one element at a time. Pretreated sample was taken as a carbon source. Peptone, yeast extract, Sodium chloride, KH_2PO_4, K_2HPO_4, Ammonium chloride, Ammonium nitrate, Magnesium chloride, Copper sulphate, Zinc chloride were some of the elements used for the process.

2.10 Plackett Burman Design:

The Plackett–Burman design was firstly employed to identify the significant variables on ethanol production from the main inorganic components, yeast extract. According to Plackett–Burman design, each variable is represented at two levels, high (+1) and low (-1). In this study, seven assigned variables together with four dummy variables were tested in 12 experiments.

$E_{Vi} = \sum y_{Vi(+)} - \sum y_{Vi(-)}/(N/2)$

where E_{Vi} represents the effect of variable i, $y_{Vi(+)}$ and $y_{Vi(-)}$ are the response of the high and low levels of variable i, respectively, N stands for number of trials. And then the standard deviation (SD) of dummies was calculated by:

$$SD = \sqrt{\sum (Ed)2}/n$$

where SD represents the effect of dummy variables, and n is the number of the dummy variables. T-test was finally performed as follows to determine the significance of these factors.

$$tvi = Evi/SD$$

Four dummy variables were studied in 12 experiments to calculate the standard error. R^2 (the coefficient of determination) was used to examine the fitness of the Plackett–Burman design. Average value of ethanol production was taken as the response. Variables with confidence levels above 90% were considered to have significant effect on ethanol production and thus were used for further optimization.

2.11 Response Surface Methodology:

Response surface method was used to optimize the effect of bacterial growth, cellulose activity and ethanol concentration for different media composition. A full quadratic polynomial regression model was used to correlate the experimental data. The established polynomial equation was used to plot three-dimensional (3-D) surfaces and visualize individual and interactive effects of the process factors on the response variables within the predefined range.

3.Results And Discussions

3.1 Sample Collection:

Allium ascalonicum leaf were collected from agricultural field and dried in hot air oven at 60°C for 3 hr and made as powder. The dried powdered sample was stored at 4°C for further studies.

Fig3.1 Fresh sample

Fig 3.2 Dried sample

3.2 Biochemical Characterization:

Allium ascalonicum leaf was a good raw material for ethanol production. The *Allium ascalonicum* leaf a rich source of carbohydrates. Anbuselvi and Balamurugan (2013) reported biochemical characterization of cassava pulp and leaves. In their work they have estimated the amount of protein content present in cassava pulp (6.8/mg) and leaves (7.0/mg). Roviero *et al*., (2015) has reported the cellulose and lignin content of sugarcane juice under biochemical characterization. The values obtained from cellulose and lignin, for sugar cane bagasse were around 48% and 34.5%.

The protein content present in *Allium ascalonicum* leaf has been estimated to have 0.043% (w/w). The leaves have been estimated to have 11.8 % of cellulose content and 0.12 % lignin content. 85 % of moisture content and 15 % of total solid contents were estimated in *Allium ascalonicum* leaf.

The biochemical constituents of *Allium ascalonicum* leaf were summarized in Table 3.1.

Table: 3.1: Biochemical composition of *Allium ascalonicum* leaf

Constituents	**%**
Total Moisture Content	85 ± 0.1
Total Solids	15 ± 0.1
Total Cellulose Content	11.8 ± 0.004
Total Protein Content	0.043 ± 0.0002
Total Nitrogen Content	0.0069 ± 0.0002
Total Lignin Content	0.12 ± 0.01

4.3 Pretreated Sample:

The sample was hydrolyzed by acid pretreatment for the removal of cellulose content combined with lignin. Acid reacts directly with the substrate and removes all the lignin content present in the sample. The lignin was removed from the residue by repeated washing with distilled water and acetone and ethanol in 1:1 ratio. The washed residue contains only cellulose content.

Fig 4.4 Dried sample

Fig 4.5 Pretreated sample

3.4 Isolation Of Cellulolytic Thermophilic Microbe From Compost Soil:

The compost soil contains the microbes which has been isolated for checking the cellulase productivity. 1 gram of sample was taken and 10 ml of distilled water was added to it. Then serial dilution was performed up to 10^{-5} dilution rate in test tubes. The serially diluted soil sample was plated by spread plate technique in CMC agar plate. Then a group of colony has been isolated from the spread plate and streak plate was performed to isolate a single colony. 6 isolates has been marked and plated in CMC agar plate.

3.5 Identification Of Cellulolytic Thermophilic Microbe:

3.5.1 Colony Morphological Identification:

Six isolates has been isolated from spread plate and plated in CMC agar plate by streak plate technique (Fig 3.6). Morphological identification was observed for the six isolates showe in Table 3.2. The isolate 1 and isolate 2 were circular in shape. Isolate 3 appeared as filamentous in shape. Isolate 4, 5 and 6 were appeared as irregular in shape. Then size of the isolate was examined for these six isolates. Isolate 1 appeared as puncti form and isolate 2 appeared as small in size. The size of the isolates 4, 5 and 6 were larger in size. On observing the elevation part the isolate 1, 3 and 5 were flat and the isolates 2, 4 and 6 were slightly raised. Margin of isolate 1 and 2 were entire and isolate 4, 5 and 6 were undulate. Isolate 3 appeared as filamentous in nature.

The texture and appearance of all the isolates was smooth and glistening in nature except isolate 3 which has rigid texture and dull appearance. Only isolate 6 was observed as pigmented (pale green) microbe where all other isolates were observed as non-pigmented. The optical property of the isolates was also noted. Isolate 3 was translucent and all the other isolates were opaque in nature.

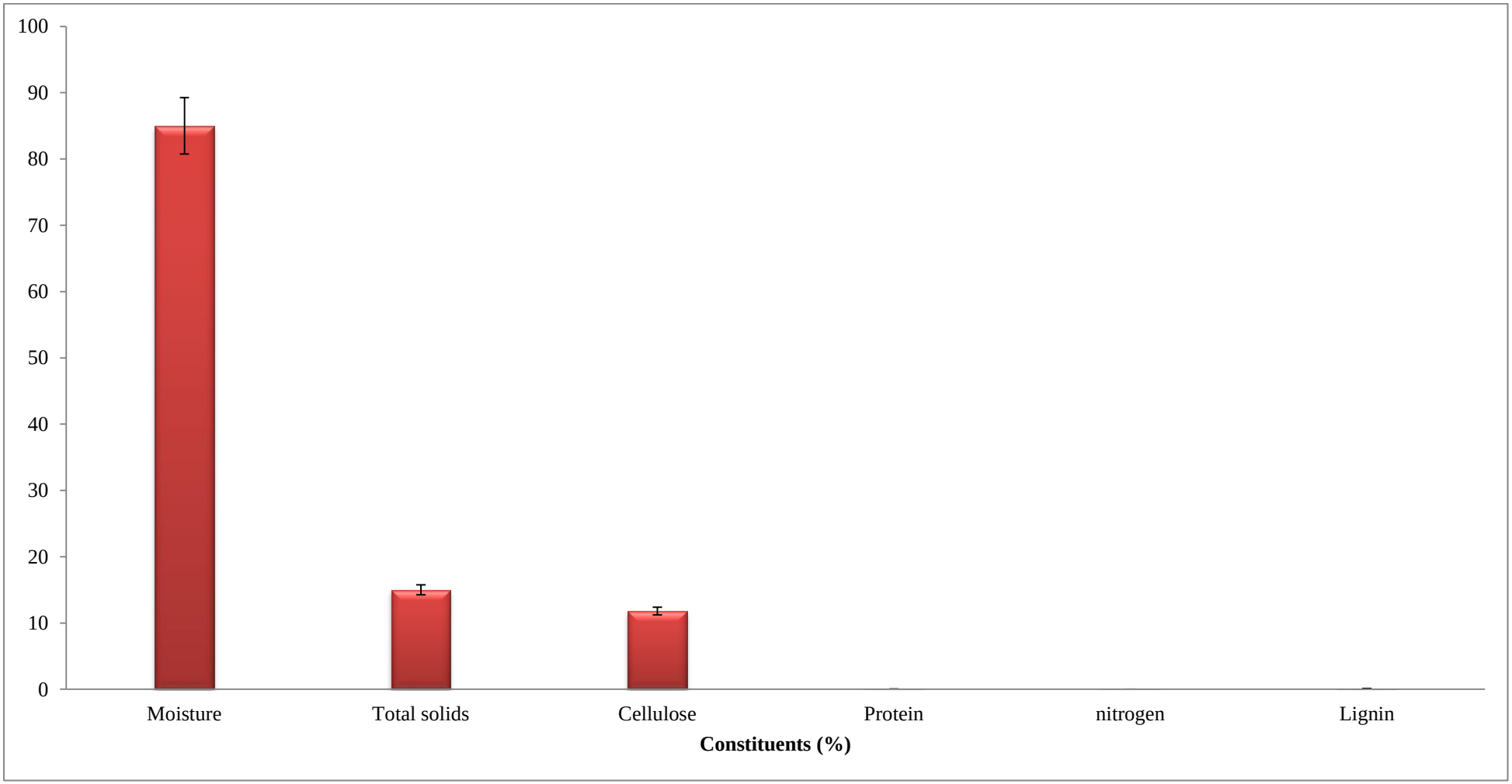


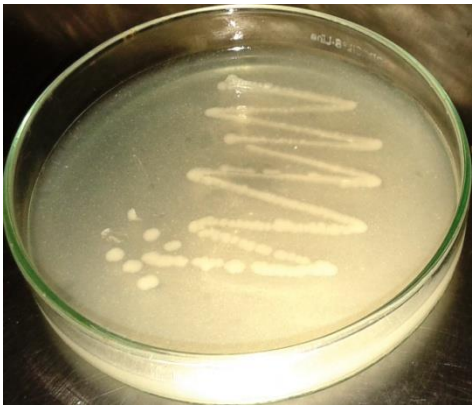
Figure 3.3 Biochemical profile of *Allium ascalonicum* leaf

a)Isolate 1

b) Isolate 2

c) Isolate 3

d) Isolate 4



e) Isolate 5

d) Isolate 6

Figure 3.6 Different bacterial isolates

Table 3.2 Morphological characteristics of isolated cellulolytic bacterium

S.No	Morphology	Observation					
		Isolate 1	**Isolate 2**	**Isolate 3**	**Isolate 4**	**Isolate 5**	**Isolate 6**
1	**Shape**	Circular	Circular	Filamentous	irregular	Irregular	Irregular
2	**Size(diameter)**	Punctiform	Small	Large	large	Large	Large
3	**Elevation**	Flat	Raised	Flat	raised	Flat	Raised
4	**Margin**	Entire	Entire	Filamentous	undulate	Undulate	Undulate
5	**Texture**	Smooth	Smooth	Rigid	smooth	Smooth	Smooth
6	**Appearance**	Glistening	Glistening	Dull	glistening	Glistening	Glistening
7	**Pigmentation**	Non Pigmented	Non pigmented	Non pigmented	Non pigmented	Non Pigmented	Pigmented (pale green)
8	**Optical property**	Opaque	Opaque	Translucent	opaque	Opaque	Opaque

3.5.2 Gram's Staining:

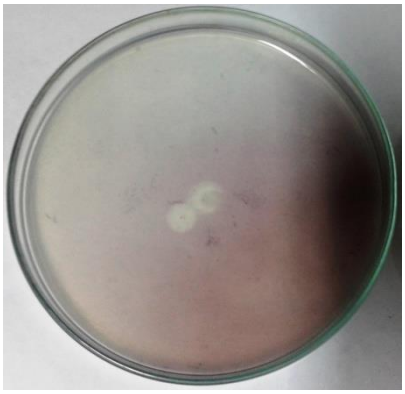
Gram's staining was performed for all the six isolates. The six isolates were smeared on a glass sli and few drops of crystal violet were added to the smear. Then the slides were washed with distilled water a then few drops of iodine solution was added and left i for few seconds. The slide was then washed w distilled water and followed by few drops of saffranin and allowed it for few minute. Finally the slide was washed with distilled water and viewed under light microscope. The first four isolates were observed to be gram negative bacteria and rod shaped. The isolate 5 was a gram positive bacteria and rod shaped. The isolate 6 was a gram negative and spherical in shape (Table 4.3).

Table 3.3 Gram's staining

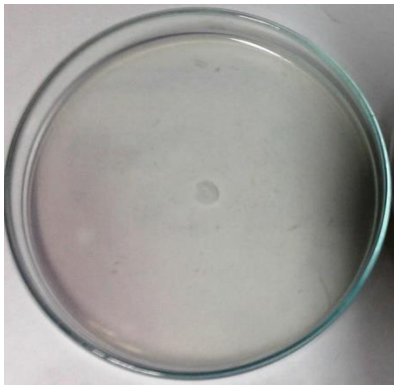
S.No	Isolate	Observation	
1	I	**(-ve)**	**Rod**
2	II	**(-ve)**	**Rod**
3	III	**(-ve)**	**Rod**
4	IV	**(-ve)**	**Rod**
5	V	**(+ve)**	**Rod**
6	VI	**(-ve)**	**Spherical**

3.5.3 Identification of cellulase producing bacteria by gram's iodine:

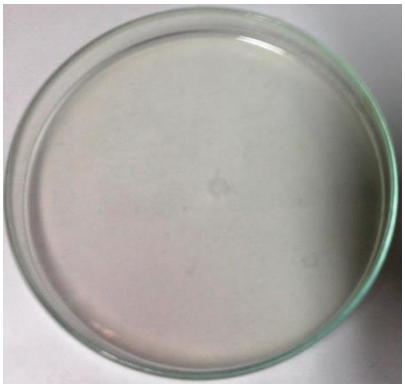
The CMC agar plates were flooded with Gram's Iodine and observed the zone of clearance which indicates cellulose hydrolysis. In Fig 4.7 clear zones were appeared around growing bacterial colonies indicating cellulose hydrolysis. The bacterial colonies having the largest clear zone were selected for identification and cellulase production in submerged system.



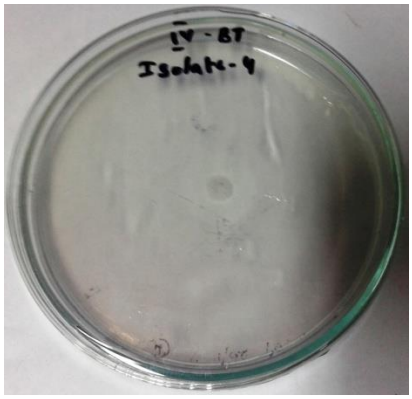
a) Isolate 1



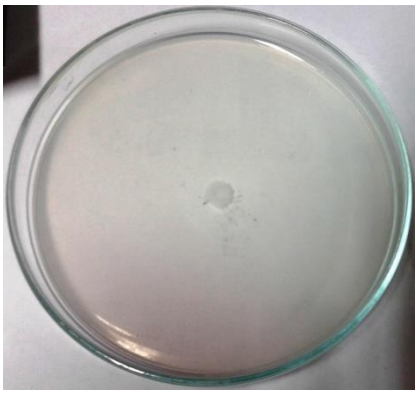
b) Isolate 2



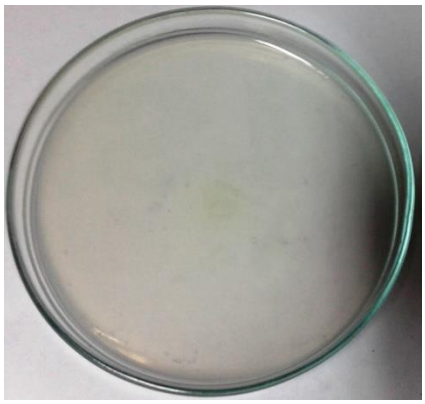
c) Isolate 3



d) Isolate 4



e) Isolate 5



f) Isolate 6

Fig 3.7 Gram's iodine test for cellulose hydrolysis

3.5.4 Cellulase assay

From the CMS hydrolysis assay cellulase activity of isolated different bacterial strains were recorded. The results shows that for the isolates 1 to 6, cellulase activity varied in the range of 0.26 to 1.57 IU/ml which is projected in Table 4.4.

Tale 3.4 Cellulase activity of isolated microorganism

	Cellulase activity(IU/ml)
Isolate 1	0.7
Isolate 2	0.23
Isolate 3	0.09
Isolate 4	1.57
Isolate 5	1.02
Isolate 6	0.26

3.6 Biochemical Tests:

Isolate 4 has the high cellulose activity and selected for the further work. This isolate 4 has been selected and biochemical tests were performed to identify the organism.

3.7 Plackett Burmann Design:

The Plackett-Burman experimental design was used in this work to screen the significant hydrolysis and fermentation parameters on bioethanol production from pretreated sample. Design-Expert (STAT-EASE Inc., Version 10(32-bit)) was applied for the experimental design and the analysis of data obtained. Eleven independent variables (Table 1) in twelve combinations were organized according to the Plackett-Burman design matrix (Table 2). For each variable, a high (+1) and a low (-1) level was tested.

Table 1: Experimental range and levels of independent variables in the Plackett-Burman experiment.

Variables	Range	
	High	Low
A.Pretreated sample	+1	-1
B.Yeast extract	+1	-1
C.Peptone	+1	-1
D.Ammonium chloride	+1	-1
E.Dipotassium hydrogen phosphate	+1	-1
F.Sodium chloride	+1	-1
G.Potassium dihydrogen phosphate	+1	-1
H.Ammonium nitrate	+1	-1

I.Magnesium chloride	+1	-1
J.Copper sulphate	+1	-1
K.Zinc sulphate	+1	-1

Table 2:Plackett-Burman design matrix representing the coded values for 11 independent variables.

Run	A	B	C	D	E	F	G	H	I	J	K	Cellulase activity IU/ml	Ethanol concentration g/L
1	1	1	-1	1	1	1	-1	-1	-1	1	-1	0.506	0.176736
2	1	1	1	-1	-1	-1	1	-1	1	1	-1	0.475	0.09468
3	-1	-1	1	-1	1	1	-1	1	1	1	-1	0.504	0.129396
4	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0.524	1.30974
5	1	-1	1	1	-1	1	1	1	-1	-1	-1	0.509	0.132552
6	1	-1	-1	-1	1	-1	1	1	-1	1	1	0.553	0.119928
7	-1	1	1	-1	1	1	1	-1	-1	-1	1	0.49	0.074166
8	-1	1	-1	1	1	-1	1	1	1	-1	-1	0.564	0.09468
9	-1	-1	-1	1	-1	1	1	-1	1	1	1	0.777	0.566502
10	-1	1	1	1	-1	-1	-1	1	-1	1	1	0.502	0.159378
11	1	-1	1	1	1	-1	-1	-1	1	-1	1	0.516	0.093102
12	1	1	-1	-1	-1	1	-1	1	1	-1	1	0.55	0.0789

3.8 Response Surface Method:

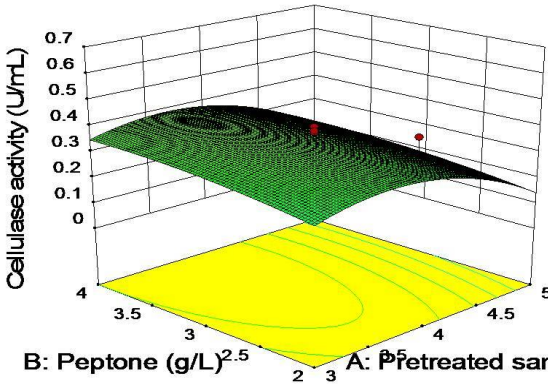
Response surface methodology (RSM) based on Box Benhen Design (BBD) was used to determine the optimum condition for maximum level of bioethanol production from the pretreated sample. The four most significant factors (pretreated sample, peptone, potassium di hydrogen phosphate and sodium chloride) were investigated at five different levels (-1, 0, 1) (Table 1) and the experimental design used for this study is shown in Table 2. All trials were performed, with the mean values of ethanol productivity considered as the response.

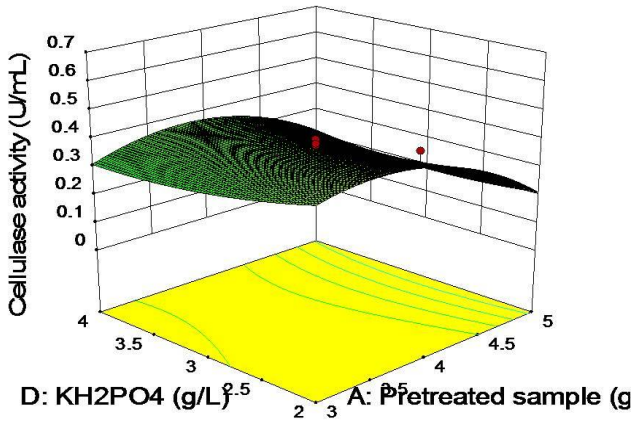
Table 1

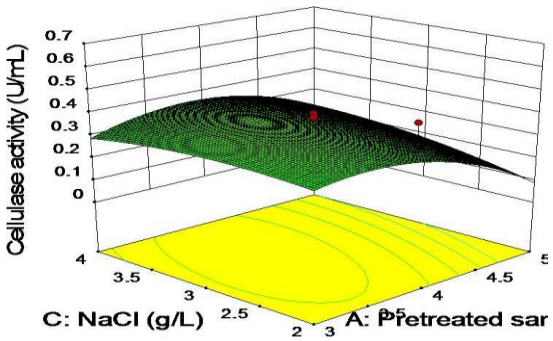
Run	P.Sample	Peptone	Nacl	KH2PO4	Cellulase activity IU/ml	Ethanol concentration g/L
1	4	3	3	3	0.316879	0.995718
2	4	5	3	3	0.503523	1.40915
3	4	3	3	3	0.399424	0.95807
4	4	3	5	3	0.463405	0.95667
5	3	4	2	2	0.566343	0.93932
6	5	2	4	4	0.493052	0.81763
7	3	4	4	4	0.120937	0.90938
8	4	3	1	3	0.0686086	0.672228
9	5	4	2	4	0.0825629	0.93143
10	3	4	4	2	0.172103	0.95825
11	5	4	4	2	0.081993	0.82827
12	4	1	3	3	0.115123	0.850722
13	4	3	3	5	0.353509	0.33927
14	5	4	4	4	0.153497	0.762174
15	5	2	2	4	0.012465	0.353472
16	3	4	2	4	0.35816	0.9865
17	5	4	2	2	0.281412	0.645582
18	3	2	4	2	0.355835	0.83156
19	3	2	4	4	0.362812	0.90974

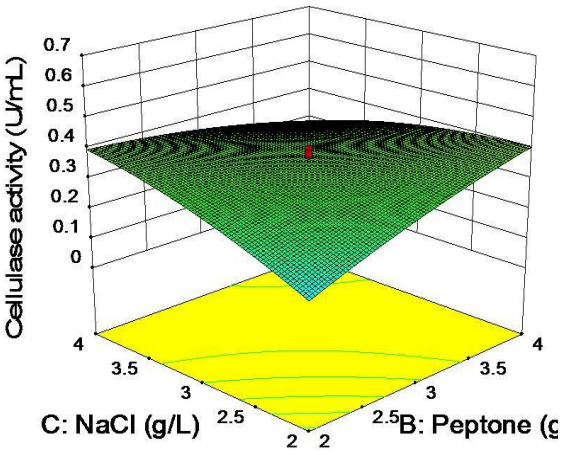
20	3	2	2	4	0.265132	0.937872
21	4	3	3	3	0.376754	0.9653
22	4	3	3	1	0.645465	0.80794
23	5	2	4	2	0.021753	0.787422
24	3	2	2	2	0.225013	0.82056
25	2	3	3	3	0.120937	0.87065
26	5	3	3	3	0.247689	0.904194
27	4	3	3	3	0.333147	0.939676
28	5	2	2	2	0.122222	0.885258
29	4	3	3	3	0.338985	0.95528
30	4	3	3	3	0.383156	0.94955

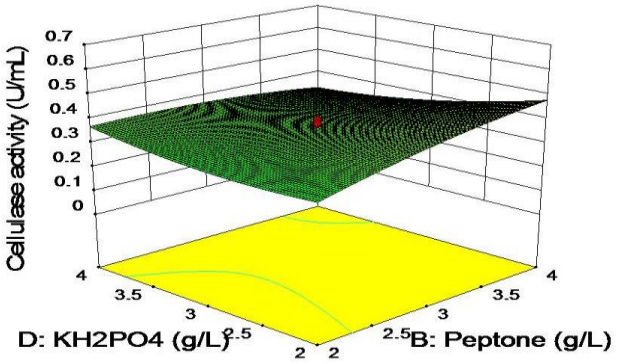
The following fig shows the RSM of cellulose activity:

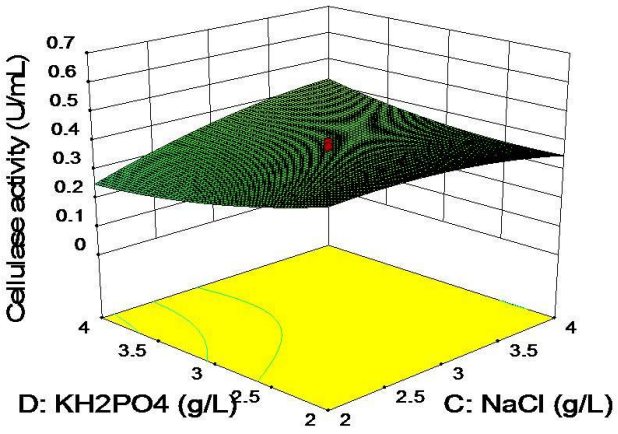




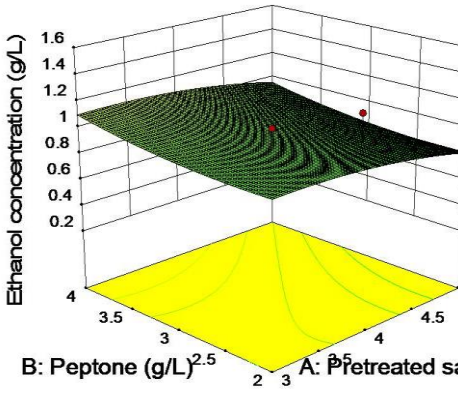


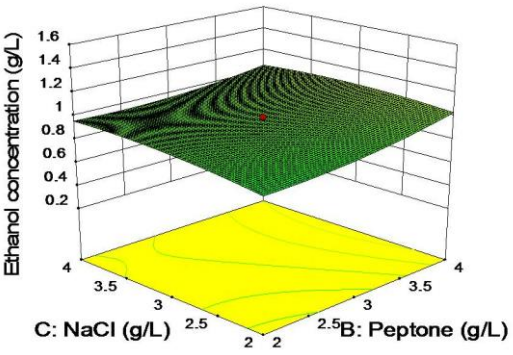


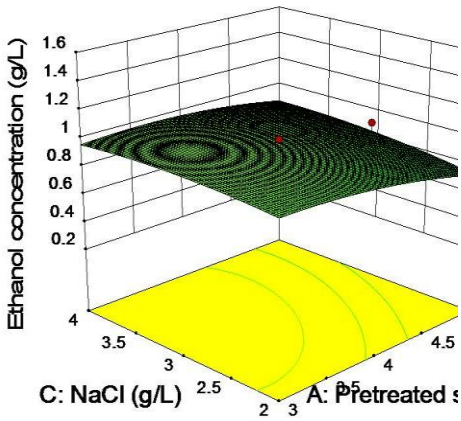




The following fig shows the RSM of ethanol concentration:


Ethanol concentration (g/L)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
4
3.5
3
2.5
2
B: Peptone (g/L)
3
3.5
4
4.5
A: Pretreated sa


Ethanol concentration (g/L)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
4
3.5
3
2.5
2
C: NaCl (g/L)
2
2.5
3
3.5
4
B: Peptone (g/L)


Ethanol concentration (g/L)
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C: NaCl (g/L)
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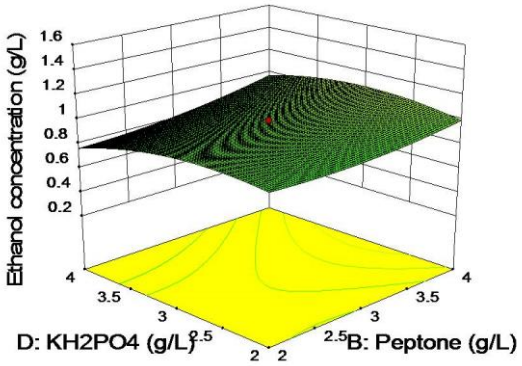
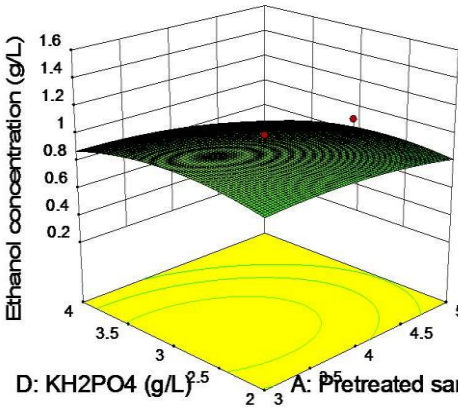

Ethanol concentration (g/L)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
4
3.5
3
2.5
2
D: KH2PO4 (g/L)
2
2.5
3
3.5
4
B: Peptone (g/L)


Ethanol concentration (g/L)
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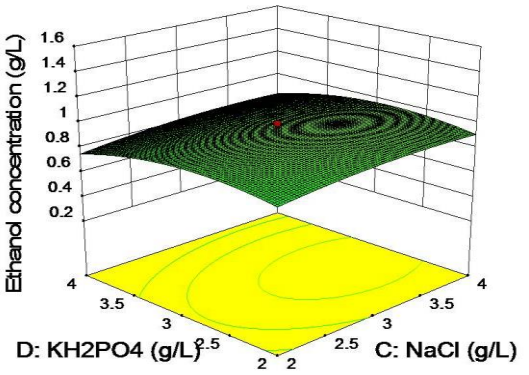
Ethanol concentration (g/L)
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D: KH2PO4 (g/L)
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C: NaCl (g/L)

4. Conclusion

The lignocellulosic feedstock is the most permissible potential source for bioethanol production. *Allium ascalonicum* leaf contains 15% cellulose on the dry weight basis, which should be the potential source for fuel production. Six different bacterial isolates were selected and screened for cellulase and ethanol production.

REFERENCES

1. Aanniz, T., Ouadghiri, M., Melloul, M., Swings, J., Elfahime, E., Ibijbijen, J., Ismaili, M. and Amar, M. (2015) 'Thermophilic bacteria in Moroccan hot springs, salt marshes and desert soils', Brazilian Journal of Microbiology Vol. 46-2, pp.443-453.
2. Acharya, A., Joshi, D.R., Shrestha, K. and Bhatta, D.R. (2012) 'Isolation and Screening of Thermophilic Cellulolytic Bacteria from Compost Piles', Scientific World, Vol. 10.
3. Acharya, S. and Chaudhary, A. (2011) 'Effect of Nutritional and Environmental factors on Cellulases Activity by Thermophilic Bacteria Isolated from Hot Spring', Journal of Scientific and Industrial Research Vol.70, pp.142-148.
4. Adiguzel, A., Ozkan, H., Baris, O., Inan, K., Gulluce, M., Sahin, F. (2009) 'Identification and characterization of thermophilic bacteria isolated from hot springs in Turkey', Journal of microbiological methods Vol.79, pp.321-328.
5. Adiguzel, A., Inan, K., Sahin, F., Arasoglu, T., Gulluce, M., Belduz, O., Baris, O. (2011) 'Molecular diversity of thermophilic bacteria isolated from Pasinler hot spring', Turk J Biol Vol.35, pp. 267-274.
6. Allais, J.J., Lopez, G.H., Kammoun, S. and Baratti, J.J. (1987) 'Isolation and Characterization of Thermophilic Bacterial Strains with Inulinase Activity', Applied And Environmental Microbiology Vol.53 No.5, pp.942-945.
7. Baharuddin, A.S., Razak, M.N.A., Hock, L.S., Ahmad, M.N., Aziz, A.S., Rahman, N.A.A., Shah, U.K.M., Hassan, M.A., Sakai, K. and Shirai, Y. (2010) 'Isolation and Characterization of Thermophilic Cellulase-Producing Bacteria from Empty Fruit Bunches-Palm Oil Mill Effluent Compost', American Journal of Applied Sciences Vol.7 (1), pp.56-62.
8. Benvenga, M.A.C., Librantz, A.F.H., Santana, J.C.C. and Tambourgi, E.B. (2015) 'Genetic algorithm applied to study of the economic viability of alcohol production from Cassava root from 2002 to 2013', Journal of Cleaner Production, pp.1-12.
9. Betiku, E. and Taiwo, A.E. (2015) 'Modeling and optimization of bioethanol production from breadfruit starch hydrolyzate vis-a-vis response surface methodology and artificial neural network', Renewable Energy Vol.74, pp.87-94.
10. Bozoglu, C., Hundur, S., Alaylar, B., Karadayi, M. and Gulluce, M. (2015) 'Isolation and Molecular Characterization of Thermophilic Bacteria with Xylanase Activity from Thermal Springs in Erzurum', Journal of Life Sciences and Technologies Vol.3, No.1.
11. Charoen, K., Prapainainar, C., Sureeyatanapase, P., Suwannaphisit, T., Wongammompitak, K., Kongkachuichay, P., Holmes, S.M., and Prapainainar, P. (2016) 'Application of Response Surface Methodology to Optimize Direct Alcohol Fuel Cell Power Density for Greener Energy Production', *Journal of Cleaner Production*.
12. Chantoom, K., Vikromvarasiri, N. and Pisutpaisal, N. (2014) 'Kinetics of Bioethanol Production from Glycerol by Enterobacter aerogenes', Energy Procedia Vol. 61, pp. 2244 – 2248.
13. Chaturvedi, V. and Verma, P. (2013) 'An overview of key pretreatment processes employed for bioconversion of lignocellulosic biomass into biofuels and

value added products', 3 Biotech Vol.3, pp.415–431.

14. Chaturvedi, A., Shukla, S., Gautam, A. and Singh, K.V (2015) 'Incidence of spore forming Clostridium sporogenes in different dairy products and their industrial and public health significance', The Pharma Innovation Journal, 3(11), pp. 30-32.
15. Corrales, R.C.N.R., Mendes, F.M.T., Perrone, C.C., Anna, C.S., Souza, W.D., Abud, Y., Bon, E.P.D.S. and Leitao, V.F. (2012) 'Structural Evaluation of Sugarcane Bagasse Steam Pretreated in the Presence of CO_2 and SO_2', Biotechnology for Biofuels Vol.5:36.
16. Das, S., Bhattacharya, A., Haldar, S., Ganguly, A., Gu, S., Ting, Y.P. and Chatterjee, P.K. (2015) 'Optimization of enzymatic saccharification of water hyacinth biomass for bio-ethanol: Comparison between artificial neural network and response surface methodology', Sustainable Materials and Technologies Vol.3, pp.17–28.
17. Desai, M.K., Survase, A.S., Saudagar, S.P., Lele, S.S., Singhal, S.R. (2008) 'Comparison of artificial neural network (ANN) and response surface methodology (RSM) in fermentation media optimization: Case study of fermentative production of scleroglucan', Biochemical Engineering Journal, Vol.41, pp. 266–273.
18. Dil, E.A., Ghaedi, M., Asfaram, A., Mehrabi, F., Bazrafshan, A.A., and Ghaedi, A.M. (2016) 'Trace determination of safranin O dye using ultrasound assisted dispersive solid-phase micro extraction: Artificial neural network-genetic algorithm and response surface methodology', Ultrasonics Sonochemistry.
19. Falter, C., Zwikowics, C., Eggert, D., Blumke, A., Naumann, M., Wolff, K., Ellinger, D., Reimer,R. and Voigt, C.A. (2015)' Glucanocellulosic ethanol: the undiscovered biofuel potential in energy crops and marine biomass', Scientific Reports Vol. 5:13722.
20. Ghati, A., Sarkar, K. and Paul,G. (2013) 'Isolation, Characterization and Molecular Identification of Esterolytic Thermophilic Bacteria from an Indian Hot Spring', Current Research in Microbiology and Biotechnology Vol. 1, No. 4,pp: 196-202.
21. Giordano, P.C., Beccaria, A.J., Goicoechea, H.C. (2011) 'Significant factors selection in the chemical and enzymatic hydrolysis of lignocellulosic residues by a genetic algorithm analysis and comparison with the standard Plackett–Burman methodology', Bioresource Technology, Vol.102, pp.10602-106010.
22. Gohel, H.R., Contractor, C.N., Ghosh, S.K., Braganza, V.J.S (2014) 'A comparative study of various staining techniques for determination of extra cellular cellulase activity on Carboxy Methyl Cellulose (CMC) agar plates', International Journal of current Microbiology and Applied science Vol.3, pp:261-266.
23. Grahovac, J., Dodic, J., Jokic, A., Dodic, S., Popov,S. (2012) 'Optimization of ethanol production from thick juice: A response surface methodology approach', Fuel, Vol.93,pp.221-228.
24. Hanly, T.J. and Henson, M.A. (2013) 'Unstructured Modeling of a Synthetic Microbial Consortium for Consolidated Production of Ethanol', The International Federation of Automatic Control pp.16-18.
25. Ho, C.Y., Chang, J.J., Chin, T.Y., Mathew, G.M., Huang, C.C. (2011) 'Establishment of functional rumen bacterial consortia (FRBC) for simultaneous biohydrogen and bioethanol production from lignocellulose', International Journal of Hydrogen Energy, Vol.36, pp.12168-12176.
26. Ighose, B.O., Adeleke, I.A., Damos, M., Junaid, H.A., Okpalaeke, K.E. and Betiku, E. (2017) 'Optimization of biodiesel production from Thevetia peruviana seed oil

by adaptive neuro-fuzzy inference system coupled with genetic algorithm and response surface methodology', Energy Conversion and Management Vol.132, pp. 231–240.

27. Jeong, S.J., and Lee, J.W. (2016) 'Optimization of pretreatment condition for ethanol production fromoxalic acid pretreated biomass by response surface methodology', *Industrial Crops and Products*, Vol.79, pp.1-6.
28. Jia, X., Liu, C., Song, H., Ding, M., Du, J., Ma, Q. and Yuan, Y. (2016) 'Design, analysis and application of synthetic microbial consortia', Synthetic and Systems Biotechnology Vol.1, pp.109–117.
29. Joyce, B.L., Zheljazkov, V.D., Sykes, R., Cantrell, C.L., Hamilton, C., Mann, D.G. J., Rodriguez, M., Mielenz, J.R., Astatkie, T., Stewart, N.C. (2015) 'Ethanol and High-Value Terpene Co- Production from Lignocellulosic Biomass of Cymbopogon flexuosus and Cymbopogon Martinii', PLOS ONE Vol.10:e0139195.
30. Kaneko, S., Mizuno, R., Maehara, T. and Ichinose, H. (2012) 'Consolidated Bioprocessing Ethanol Production by Using a Mushroom' National Food Research Institute.
31. Kasana, R.C., Salwan, R., Dhar, H., Dutt, S. and Gulati, A. (2008) 'A Rapid and Easy Method for the Detection of Microbial Cellulases on Agar Plates Using Gram's Iodine', Curr Microbiol Vol.57, pp.503–507.
32. Khoobbakht, G., Najafi, G., Karimi, M. (2015) 'Optimization of operating factors and blended levels of diesel, biodiesel and ethanol fuels to minimize exhaust emissions of diesel engine using response surface methodology', Applied Thermal Engineering.
33. Klein, M., Griess, O., Pulidindi, I.N., Perkas, N. and Gedanken, A. (2016) 'Bioethanol production from Ficus religiosa leaves using microwave Irradiation', Journal of Environmental Management Vol.177, pp 20-25.
34. Koma, O.S. and Fakunle, F.A. (2014) 'Biological and Chemical Evaluation of Leaf Extracts of *Dalbergia saxatilis* Hook' , Scholars Academic Journal of Pharmacy Vol.3(1), pp.61-65.
35. Laghari, S.M., Isa, M.H., Abdullah, A.B., Laghari, A.J. and Saleem, H. (2014) 'Microwave Individual and Combined Pre-Treatments on Lignocellulosic Biomasses', IOSR Journal of Engineering Vol.04, pp.14-28.
36. Lazuka, A., Auer, L., Bozonnet, S., Morgavi, D.P., O'Donohue, M., Hernandez-Raquet, G. (2015) 'Efficient anaerobic transformation of raw wheat straw by a robust cow rumen-derived microbial consortium', Bioresource Technology.
37. Li, K., Qin, J.C., Liu, C.G. and Bai, F.W. (2016) 'Optimization of pretreatment, enzymatic hydrolysis and fermentation for more efficient ethanol production by Jerusalem artichoke stalk', Bioresource Technology.
38. Lima, M.A., Lavorente, G.B., Silva, H.D.KP., Bragatto, J., Rezende, C.A., Bernardinelli, O.D., Azevedo, E.D.R., Gomez, L.D., Mason, S.M.Q.J., Labate, C.A. and Polikarpov, I. (2013) 'Effects of pretreatment on morphology, chemical composition and enzymatic digestibility of eucalyptus bark: a potentially valuable source of fermentable sugars for biofuel production', Biotechnology for Biofuels Vol.6:75.
39. Lv, W. and Yu, Z. (2012) 'Isolation and Characterization Of Two Thermophilic Cellulolytic Strains Of Clostridium Thermocellum from a Compost Sample', Journal of Applied Microbiology Vol.114.
40. Madden, R.H. (1983) 'Isolation and Characterization of Clostridium stercorarium sp. nov., Cellulolytic Thermophile',

Internarionai Journal of Systematic Bacteriology Vol.33, No.4, pp.837-840.

41. Mazzoli, R. (2012) 'Development of microorganisms for cellulose-biofuel consolidated bioprocessings: metabolic engineers' tricks' Computational and Structural Biotechnology Journal Vol.3.
42. Mistry, T. B., Shaikh, N.M., Rana, H.N. and Patel, N.D. (2016) 'Isolation and Screening of Cellulase Producing Thermophilic Bacteria from Compost Piles and Optimization of Cellulase Production', International Journal of Advanced Biotechnology and Research Vol.7, pp.64-76.
43. Mohagheghi, A., Grohmann, K., Himmel, M., Leighton, L. and Updegraff, D.M. (1986) 'Isolation and Characterization of Acidothermus cellulolyticus gem nov., sp. nov., a New Genus of Thermophilic, Acidophilic, Cellulolytic Bacteria', International Journal Of Systematic Bacteriology Vol. 36, No. 3, pp.435-443.
44. Mohammadzadeha, A., M. Ramezania, M., Ghaedi, A.M. (2015) 'Synthesis and characterization of Fe2O3–ZnO–ZnFe2O4/carbon nanocomposite and its application to removal of bromophenol blue dye using ultrasonic assisted method: Optimization by response surface methodology and genetic algorithm', Journal of the Taiwan Institute of Chemical Engineers,pp.1-10.
45. Mshandete, A.M., Bjornsson, L., Kivaisi, A.K., Steven, M., Rubindamayugi, T. and Mattiasson, B. (2008) 'Effect of aerobic pre-treatment on production of hydrolases and volatile fatty acids during anaerobic digestion of solid sisal leaf decortications residues', African Journal of Biochemistry Research Vol.2 (5), pp. 111-119.
46. Muhammad, Y., Hadi, B.A., Bello, A.M. and Dogarai, B.B.S.(2016) 'Bioethanol Production from Neem Tree Leaves *(Azadirachtaindica*) Using Saccharomyces cerevisiae as Fermenting Agent', IOSR Journal of Applied Chemistry Vol-9, pp.32-37.
47. Nayebzadeh, H., Saghatoleslami, N., Tabasizadeh, M. (2016) 'Optimization of the activity of KOH/calcium aluminate nanocatalyst for biodiesel production using response surface methodology', Journal of the Taiwan Institute of Chemical Engineers, pp.1-8.
48. Nuwamanya, E., Karltun, L.C., Kawuki, R.S. and Baguma, Y. (2012) 'Bio-Ethanol Production from Non-Food Parts of Cassava (Manihot esculenta Crantz)', AMBIO Vol. 41, pp.262–270.
49. Panda, K.M., Sahu, M.K. and Tayung, K. (2013) 'Isolation and characterization of a thermophilic Bacillus sp. With rotease activity isolated from hot spring of Tarabalo, Odisha, India', Iranian Journal of Microbiology Vol.5, pp.159-165.
50. Pereira, S.C., Maehara, L., Machado, C.M.M. and Farinas, C.M. (2015) '2G ethanol from the whole sugarcane lignocellulosic biomass', Biotechnology for Biofuels Vol.8.
51. Petti, C., Ware, A.H.E., Tateno, M., Kushwaha, R., Shearer, A., Downie, B. A., Crocker, M. and Bolt. S.D. (2013) 'Sorghum Mutant RG Displays Antithetic Leaf Shoot Lignin Accumulation Resulting in Improved Stem Saccharification properties', Biotechnology for Biofuels Vol.6:146.
52. Prasad, P., Singh, T. and Bedi, S. (2014) 'Enzymatic Screening and Characterization of Cellulolytic Actinomycetes Isolated from Soil samples collected from Patna District in Bihar, India', International Journal of Current Research and Academic Review Vol.2, pp.60-71.
53. Prasad, P., Singh, T. and Bedi, S. (2014) 'Characterization of a novel thermophilic cellulase producing strain Streptomyces matensis strain St-5', International Journal

of Current Microbiology and Applied Science Vol.3, pp.74-88.

54. Ramos, L.P. (2003) 'The Chemistry Involved In The Steam Treatment Of Lignocellulosic Materials', Quim. Nova Vol. 26, No. 6, 863-871.
55. Raud, M., Kesperi, R., Oja, T., Olt, J. and Kikas, T. (2014) 'Utilization of urban waste in bioethanol production: potential and technical solutions', Agronomy Research Vol.12 (2), pp.397–406.
56. Sazci, A., Radfor, A. and Erenle, K. (1986) 'Detection of cellulolytic fungi by using Congo red as an indicator: a comparative study with the dinitrosalicyclic acid reagent method', Journal of Applied Bacteriology Vol.61, pp.559-562.
57. Simic, V.M., Rajkovic, K.M., Stojicevic, S.S., Kovic, D.T.V., Nikolic, N.C., Lazic, M.L., Karabegovic, I.T. (2016) 'Optimization of microwave-assisted extraction of total polyphenolic compounds from chokeberries by response surface methodology and artificial neural network', Separation and Purification Technology, Vol.160, pp.89-97.
58. Sissons, C.H., Sharrock, K.R., Daniel, R.M. and Morgan, H.W. (1987) 'Isolation of Cellulolytic Anaerobic Extreme Thermophiles from New Zealand Thermal Sites', Applied And Environmental Microbiology Vol.53 No.4, pp. 832-838.
59. Stanek, J., Mairinger, S., Wanek, T., Kunter, C., Muller, M., Langer, Langer, O. (2015) 'Automated radiosynthesis of ciprofloxacin', Applied Radiation and Isotopes, Vol.99, pp.133-137.
60. Stathopoulou, P.M., Savvides, A.L., Karagouni, A.D., and Hatzinikolaou, D.G. (2013) 'Unraveling the Lipolytic Activity of Thermophilic Bacteria Isolated from a Volcanic Environment', BioMed Research International.
61. Tutt, M., Kikas, T. and Olt, J. (2012) 'Influence of different pretreatment methods on bioethanol production from wheat straw', Agronomy Research Biosystem Engineering Special Vol.1, pp.269-276.
62. Upadhyaya, S.K., Manandhar, A., Mainali, H., Pokhrel, A.R., Rijal, A., Pradhan, B. and Koirala, B. (2012) 'Isolation and Characterization of Cellulolytic Bacteria from Gut of Termite', Rentech Symposium Compendium, Vol.1.
63. Varma, S.V., Narayanan, H.T.R., Kamala, K., Shivashankar, B. and Patil, J. H. (2013) 'Comparision of Acidic and Basic Pretreatment on Saccharification of Water Hyacinth', International Journal of Innovative Research in Science, Engineering and Technology Vol.2, pp.2319-8753.
64. Vikromvarasiria, N., Laothanachareonb, T., Champredab, V. and Pisutpaisala, N. (2014) 'Bioethanol Production from Glycerol by Mixed Culture System', Energy Procedia Vol. 61, pp. 1213 – 1218.
65. Watkins, D., Nuruddin, Md., Hosur, M., Narteh, A.T. and Jeelani, S. (2014) 'Extraction and Characterization of Lignin from Different Biomass Resources', Journal of Materials Research and Technology Vol. 4(1), pp.26-32.
66. Yang, W., Meng, F., Peng, J., Han, P., Fang, F. and Ma, L. and Cao, B. (2014) 'Isolation and identification of a cellulolytic bacterium from the Tibetan pig's intestine and investigation of its cellulase production', Electronic Journal of Biotechnology Vol.17, pp.262-267.
67. Zambare, V., Zambare, A., Muthukumarappan, K. and Christopher, L.M., (2011) 'Biochemical characterization of thermophilic lignocellulose degrading enzymes and their potential for biomass bioprocessing', International Journal of Energy and Environment Vol.2, pp.99-112.