BIOETHANOL PRODUCTION FROM LIGNOCELLULOSIC WASTE BY SUBMERGED FERMENTATION

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ABSTRACT

In the present study three lignocelluosic wastes viz., SugarcaneBaggase, Ragi husk and Broad bean peels were used as substrates for production of Bioethanol using successive saccharification and fermentation Process. In addition 3 pre-treatment methods (Hot Water, NaOH and & dilute sulphuric acid (1.0N) were evaluated in the study. Saccharification was carried out usinf two different enzymes viz., pectinase and α-amylase. Three yeast species namely, *Saccharomyces cerevisiae, Kluveromyces marixianus and Zymomonas mobilis* were used for the process of fermentation. Maximum ethanol production was obtained in Ragi husk and Sugarcanebaggase when NaOH pretreatment was used in combination with pectinase and α-amylase enzyme respectively for hydrolysis and *Kluveromyces marixianus* for fermentation at 50° C. It recorded about 16.44% and 14.66% of ethanol production respectively. In case of broad bean peels maximum ethanol yield of 8.45% was obtained in hot water pretreatment followed by hydrolysis with pectinase and fermentation using *Zymomonas mobilis* at 25° C.

Keywords: Bioethanol, Ragi husk, Sugarcane bagasse, Broad bean peel, submerged state fermentation.

INTRODUCTION

The worldwide interest in obtaining sustainable energy for the future motivates the search for other means of energy production, as fossil fuels are becoming increasingly scarce[1]. The world ethanol production has reached about 51,000 million litres, being the USA & Brazil the first producers and India stands fourth among the top fuel ethanol producers[2]. Ethanol contains 35% oxygen, which results in a complete combustion of fuel and thus lowers the emission of harmful gases. Moreover, ethanol production uses energy from renewable sources only; hence, no net carbon dioxide is added to the environment, thus reducing green-house gas emissions. It has also been well established now that ethanol increases the octane number, decreases the Reid vapor pressure and produces fuel with clean burning characteristics[3]. Moreover, neat (unblended) ethanol can be burned with greater efficiency, and is thought to produce smaller amounts of ozone precursors (thus decreasing urban air pollution), and is particularly beneficial with respect to low

net CO_2 put into the atmosphere[4]. Further United States Environmental protection Agency (EPA) announced the beginning of regulatory action to eliminate MTBE in gasoline because it is a toxic chemical compound and has been found to contaminate group water. Hence, the demand for ethanol could increase further if MTBE is eliminated from gasoline[5].

Since the price of feedstock contributes more than 55% to the production cost, inexpensive feed stocks such as lignocellulosic biomass and agri-food wastes, are being considered to make bioethanol competitive in the open market[6]. The production of ethanol from comparatively cheaper source of raw materials using efficient fermentative microorganisms is the only possible way to meet the great demand for ethanol in the present situation of energy crisis[7]. Lignocellulosic wastes are produced in large amounts by different industries including forestry, pulp and paper, agriculture and food, in addition to different wastes from municipal solid waste (MSW), and animal wastes[8].

The lignocellulosic materials are formed by three structural polymers; cellulose, hemicelluloses and lignin and small quantities of other compounds. Among these components, carbohydrates (cellulose and hemicelluloses) can be saccharified and eventually fermented to obtain bioethanol. The structural complexity of the lignocellulosic materials hinders enzymatic hydrolysis for what their conversion to bioethanol requires a pretreatment step that can be carried out by different techniques, using alkalis, acids, heat, pressure, solvents, etc[9]. Increased yield of ethanol production by microbial fermentation depends on the use of ideal microbial strain, appropriate fermentation substrate and suitable process technology. Both bacteria and fungi can produce glucanases (cellulases) that hydrolyze of lignocellulosic materials[10]. Lignocellulolytic microorganisms, especially fungi, have attracted a great deal of interest as biomass degraders for large-scale applications due to their ability to produce large amounts of extracellular lignocellulolytic enzymes. In most of these studies the preferred candidate for industrial production of ethanol has been *S.cerevisiae*. Yeast also has the ability to produce ethanol which is not contaminated by other products from the substrate[11]. *Zymomonas mobilis*, the gram negative anaerobic bacteria, notable for bioethanol production. Surpass yeast in some aspects, degrades sugar to pyruvate using the E.D pathway which results in the production of ethanol and carbondioxide[12].

Kluveromyces marixianus is a thermotolerant yeast that has been explored for potential use in biotechnological application such as production of biofuels, single cell proteins, enzymes and other heterologous proteins. *K. marixianus* produces high percentage of ethanol at 50°C[13]. In the present study three lignocellulosic wastes were used as substrate. Sugarcane bagasse (SCB) is emerging as an attractive feedstock for bioethanol production. SCB is one of the major lignocellulosic materials found in great quantities to be especially in tropical countries; Bagasse is primarily composed of lignin (20-30%), cellulose (40-45%) and hemicelluloses (30-35%). Because of its lower ash content (1.9%), bagasse offers numerous

advantages compared with other agro-based residues such as paddy straw (16%), rice straw (14.5%) and wheat straw (9.2%) [14].

For every 100 tons of Sugarcane crushed, a Sugar factory produces nearly 30 tons of wet bagasse [15]. Secondly Ragi being the third most important millet in India and Karnataka is the top producer sharing 58% in India's export of this crop[16].Ragi produces both straw and rice husks at the processing plant which can be conveniently and easily converted into energy[17]. In this current study we focus on Ragi husk as it is a complete lignocellulosic waste which is usually dumped or burnt to ashes when compared to the straw which is mostly used as a fodder for the livestock. The third raw material *Vicia faba*, also known as the broad bean, fava bean, faba bean, field bean, bell bean, or tic bean, is a species of bean belongs to the family Fabaceae, native to North Africa, southwest and south Asia. Oligosaccharides such as stachyose, raffinose and verbascose are prevalent in faba bean. These molecules contain glucose and galactose residues which can be fermented for the production of ethanol[18].

MATERIALS & METHODS:

The raw materials Sugarcane bagasse, Ragi husk and Broad bean peels were collected from local sugarcane juice centre, mill and Krishanarajapuram market, Bangalore respectively. 25g of each of the samples was weighed and transferred to five sterilized culture bottles (duplicates was also maintained).

PRETREATMENT:

The samples were subjected to three different pre-treatments viz., dilute acid pre-treatment (DAP), hot water pre-treatment (HP) & alkaline pre-treatment (AP). DAP was carried out using dilute sulphuric acid (1.0N). The samples were soaked in the dilute acid overnight and used for further analysis. In HP, samples were autoclaved at 121ºC at 15 psi pressure for 30 minutes. It was then cooled to room temperature and used for further analysis. For AP 2% NaOH was used where the samples were soaked in 2% NaOH overnight and used for further analysis.

ENZYMATIC SACCHARIFICATION:

Each of the three samples was subjected to two different enzymes i.e. α-amylase and pectinase separately. 0.2 gms of each enzyme were added into the respective bottles and incubated at RT (25°C) for a period of 48hrs.

FERMENTATION:

After enzyme saccharification, the three different microorganisms were introduced to the pre-treated and saccharified samples in the following manner. Out of the five bottles used for each set, *Saccharomyces cerevisiae* was introduced into first bottle, *Kluyveromyces marixianus* (MTCC 4136) was introduced into second and third bottle and *Zymomonas mobilis* (MTCC 2427) was introduced into the fourth bottle. No microorganism was introduced into the fifth bottle which was maintained as control. Replicates were maintained for all the treatments.

The culture bottles were then allowed to ferment at different temperatures, i.e., the bottles inoculated with *Saccharomyces cerevisiae, Kluyveromyces marixianus* (MTCC 4136) and *Zymomonas mobilis* (MTCC 2427) cultures were stored at room temperature (25°C). Another bottle which was also inoculated with *Kluyveromyces marixianus* (MTCC 4136) culture was stored at 50°C in an incubator. The samples were allowed to ferment for 11 days.

ETHANOL RECOVERY:

After 11 days of incubation the samples were filtered aseptically using a filter paper supported on a funnel. This ensured the stopping of fermentation by separating the fermented ethanol from the cellular biomass and the substrate (saccharified lignocelluloses). The filtrate was used for further quantification.

CONTROL USED:

Separate blanks were set up without the addition of microorganisms and enzymes for fermentation.

CONTENT ANALYSIS:

Two main tests were conducted under content analysis at various stages of the fermentation experiment. The reducing sugar content was measured using the dinitro salicylic acid test (DNS test) and the percentage of ethanol was determined by potassium dichromate and potassium iodide titration method. Reducing sugar content of the five samples was analysed initially before pre-treatment, after pre-treatment, after enzyme saccharification, after culture addition and after ethanol fermentation. Ethanol percentage was estimated initially before pre-treatment, after enzyme addition and after the fermentation process.

$$2Cr_2O_7^{2-} + 16H^- + 3C_2H_2OH \longrightarrow 4Cr^{3+} + 11H_2O + 3CH_3COOH$$

1 mole of the dichromate ≡ 3 moles of iodine ≡ 6 moles of Na_2SO_3

2 moles of dichromate ≡ 3 moles of ethanol ≡ 46 g of ethanol

Moles (dichromate) to oxidize alcohol = moles added – moles spent by sodium thiosulphate
Volume of alcohol in essay = 46g × Moles (alcohol)

RESULTS AND DISCUSSION:

In the current study, attempts were made to standardize various parameters such as pretreatment, concentration of various enzymes required and the efficiency of different yeast species on the successful fermentation process to carry out the conversion of these lignocellulosic wastes into ethanol.

Pretreatment:

Owing to the structural characteristics of lignocellulosic biomass (LB), pretreatment is an essential step for obtaining potentially fermentable sugars in hydrolysis step [19]. Three methods of pretreatment were carried out namely hot water pretreatment, dilute acid (0.2N H_2SO_4) and alkali treatment (2% NaOH), among which NaOH pretreatment at 50°C showed higher reducing sugar levels and ethanol yield for Sugarcane bagasse and Ragi husk while hot water treatment gave better ethanol yield when broad bean peels. Pretreatment of the lignocellulosic residues is necessary because hydrolysis of non-pretreated materials is slow and results in low product yield. Some pretreatment methods increase the pore size and reduce the crystallinity of cellulose [21]. Importance of pretreatment of the lignocellulosic biomasses (sugarcane Bagasse, rice straw and wheat straw) in ethanol production from these biomasses using strain of *Sacchromyces cervisiae* in submerged fermentation was reported earlier[22]. Pretreatment with NaOH increases the digestibility cellulose from 14 to 55% while decreasing the lignin content from 25 to 20% [23]. Similarly in the current study NaOH was found to be effective for Sugarcane bagasse and Ragi husk where maximum ethanol yield was obtained at a higher temperature (50°C). Increased susceptibility of lignocellulosics by alkali pretreatment might be due to the removal of lignin seal and swelling of the substrates in an alkaline condition, which might increase accessibility of the substrate to enzymatic attack [24]. Similarly for broad bean peels which have low lignin content, NaOH pretreatment was not effective when compared to hot water and dilute acid treatment. Thus, the LHW or thermo hydrolysis may be a promising pretreatment that presents elevated recovery rates of pentoses which does not generate inhibitors[25].

Hot water pretreatment and dilute acid pretreatment was not found to be effective in case of sugarcane bagasse and ragi husk. This may be due to the lower concentration of the acid and duration of hot water treatment used in the current study.

Enzymatic hydrolysis and Fermentation:

Enzymatic hydrolysis is regarded today as the most promising approach to liberating fermentable sugars in an energy-efficient way from the carbohydrates found in lignocelluloses in order to produce ethanol [20]. In the current study two different enzymes α –amylase and pectinase were used among which significant removal of lignin from Sugarcane residue was obtained from α –amylase whereas pectinase enzyme gave

better yield of reducing sugar and ethanol for Ragi husk. In case of Broad bean peel both the enzymes gave similar results.

Reducing sugar content and Ethanol production:

Sugarcane bagasse:

In the present study Sugarcane bagasse acted upon by α-amylase enzyme showed a gradual increase in reducing sugar in case of control and KM 50°C while decreased in SC 25°C, ZM 25°C and KM 25°C. Upon culture addition it was observed that reducing sugars (reducing sugar) showed a narrow decrease due to the action of the micro-organisms on the hydrolysed cellulose and lignin components which convert these simple sugars to ethanol. At the end of 11th day of fermentation the RS estimation recorded a reduction in sugar level in all the bottles except the control which showed an increase from 0.21 to 0.28 [TABLE 1]. Similar to α-amylase, the action of pectinase enzyme on Sugarcane bagasse showed a gradual increase in reducing sugar in case of control, ZM 25°C and KM 50°C while decreased in SC 25°C, ZM 25°C and KM 25°C. The reducing sugar yield obtained upon culture addition seemed to increase only in SC 25°C while decreased with other organisms and the reducing sugar recorded on 11th day of fermentation was found to be similar as obtained with amylase enzyme where only control showed an increase in reducing sugar yield [TABLE 1].

Sugarcane bagasse showed the presence of 3.66% of ethanol initially. In the reaction containing amylase enzyme, there was a significant increase in the ethanol produced following fermentation reaction where the highest percentage of ethanol yield recorded was 14.66% at 50°C with NaOH pretreatment in the presence of *Kluveromyces marixianus.* When Kluveromyces *marixianus* was maintained at 25°C with α-amylase enzyme, a better yield of only 10.02% of ethanol was obtained. When *Saccharomyces cerevisiae* was used in combination with amylase enzyme, the sample maintained at 25°C with NaOH pretreatment showed maximum yield with 12.36% of ethanol. Bagasse treated with *Zymomonas mobilis* in combination with amylase enzyme, maintained at 25°C after NaOH pretreatment showed 9.14% of ethanol. The Control maintained gave an ethanol yield of 2.91% (Table 2).

Thus the highest percentage of ethanol (14.66%) with Sugarcane bagasse was obtained upon pretreatment with NaOH and *Kluveromyces marixianus* maintained at 50°C with amylase enzyme and the lowest was 7.59% obtained upon pretreatment with NaOH and *Saccharomyces cerevisiae* maintained at 25°C with pectinase enzyme.

Earlier it was reported that ethanol yield by immobilized cells of *Kluveromyces thermotolerans* are lower than that of free cells, which they supposed to be due to mass transfer limitations of nutrients due to the formation of barrier when cells are immobilized [26]. Similarly in the current study better ethanol yield was

obtained when the culture were allowed to react in free cell state by submerged fermentation. It was also noted that the amount of ethanol content increased with the increase in fermentation time [27].

RAGI HUSK:

In the present study with Ragi husk, the initial reducing sugar concentration during pretreatment was found to be moderate in all bottles except SC 25°C which was seemingly high, upon action of α-amylase enzyme we could observe a gradual increase in reducing sugar in all bottles except in SC 25°C where the sugar concentration decreased. Upon culture addition, it was noticed that the sugar yield increased further only in control, while reduced in the other bottles. On the 11th day of fermentation the reducing sugar was found to be least in KM 50°C with 0.32, hence gave a higher ethanol yield when compared to SC 25°C, ZM 25°C and KM 25°C. A reduction in sugar level was seen in all the bottles except the control which showed an increase from 0.67 to 0.72 [Table 2]. On the 11th day of fermentation reducing sugar was found to be similar as obtained with amylase enzyme where only control showed an increase in reducing sugar yield and in the other bottles it was significantly reduced [Table 3].

Ragi husk substrate showed the initial ethanol concentration of 2.29%. In the reaction containing **α**-amylase and pectinase enzyme at 50°C in presence of *Kluveromyces marixianus* with NaOH pretreatment, there was significant increase in ethanol concentration with maximum yield of 14.94% and 16.44% respectively. *Kluveromyces marixianus* maintained at 25°C showed highest ethanol yield of 14.78% with reaction containing pectinase enzyme with NaOH pretreatment. The lowest bioethanol yield of 10.92% was obtained with *Kluveromyces marixianus* maintained at 25°C with reaction containing **α**-amylase enzyme with NaOH pretreatment. *Zymomonas mobilis* maintained at 25°C with reaction containing **α**-amylase enzyme and pectinase enzyme with NaOH pretreatment showed better bioethanol production of 13.30% and 13.56% respectively.

The reaction containing *Saccharomyces cerevisiae* and pectinase enzyme at 25°C showed maximum ethanol yield of 15.40%, while the reaction containing *Saccharomyces cerevisiae* and amylase enzyme at 25°C showed lowest ethanol yield of 9.02% only [Table 4]. It was reported previously that alkaline pretreated corn cobs, corn husks, and corn stalks produced ethanol with an overall 90% efficiency [29], Similarly in our study Ragi husk was used instead of rice straw and corn cobs, corn husks, & corn stalks which upon submerged fermentation for a period of 11 days gave a better ethanol yield of 15.40%, with *Saccharomyces cerevisiae* and pectinase enzyme at 25°C.

BROAD BEAN PEEL:

All the three pretreatment were showing increased reducing sugar concentration in Broad bean peel. Upon action of α-amylase enzyme it was observed that a gradual increase in reducing sugar was recorded in all bottles except SC 25°C (NaOH pretreated), SC 25°C and KM 50°C (hot water treatment). On the 11th day of fermentation the reducing sugar had decreased in all bottles except ZM 25°C & KM 25°C (NaOH treatment) whereas increase in reducing sugar was observed. In case of control the reducing sugar increased from 1.67 – 1.89 [Table 5]. Similarly with the action of pectinase enzyme broad bean peel showed a gradual increase in reducing sugar in all bottles except ZM 25°C ,KM 25°C and KM 50°C (acid pretreatment)and ZM 25°C & KM 50°C (NaOH pretreatment) in which there was a slight reduction in reducing sugar was noted . The reducing sugar yield obtained upon culture addition seemed to increase only in SC 25°C, KM 25°Cand KM 50°C (acid treated) and ZM 25°C (NaOH treatment) while decreased with all the other organisms. Reducing sugar obtained on the 11th day of fermentation was found to be similar with α-amylase enzyme where only control and KM 25°C (hot water pretreated) showed an increase in reducing sugar yield while in the other bottles it was significantly reduced [Table 5].

An initial ethanol concentration of 2.70% was recorded with Broad bean peel. Addition of α-amylase enzyme in the presence of *Zymomonas mobilis* maintained at 25°C with hot water treatment showed maximum ethanol yield of 8.45% and the lowest yield obtained was with the same enzyme and pretreatment but in the presence of *Saccharomyces cerevisiae* maintained at 25°C. A higher yield of 7.76% of ethanol was obtained from the fermented sample containing pectinase enzyme in the presence of *Zymomonas mobilis* maintained at 25°C with dilute acid pretreatment, while lowest yield of 6.03% was recorded with the sample containing amylase enzyme and *Kluveromyces marixianus* maintained at 50°C with dilute acid pretreatment.

In the reaction containing α-amylase enzyme and *Zymomonas mobilis* maintained at 25°C, the highest ethanol yield was 8.02% with NaOH pretreatment while the lowest yield was obtained from the fermented sample containing *Kluveromyces marixianus* maintained at 25°C with hot water treatment which is 3.95%.[Table 6].

In the present study *Saccharomyces cerevisiae* gave a better yield of ethanol with hot water pretreatment in combination with pectinase enzyme which was not that significant when compared to *Zymomonas mobilis* which gave a yield of 8.02% with NaOH pretreatment. Similar to the previous reports the broad bean peels maybe made of pentose sugars which are not fermentable by yeast, hence genetically modified strain yeast can be chosen for complete saccharification of the simple sugars to obtain a higher yield of ethanol.

CONCLUSION

Based on the current study it can be concluded that the waste substances such as lignocellulosic wastes can be utilized for the production of ethanol in a shorter span of time and in an economically cheaper way. Further immobilized enzymes can be employed for the production enabling the easy recovery of the enzymes. Ethanol percentage can be further increased by employing certain reagents that can help in increasing the saccharification surface area which would ultimately increase the ethanol yield. The protocols can further be standardized so as to increase the yield of bioethanol relatively in a higher amount.

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TABLE 1: Reducing Sugar content in Sugarcane bagasse acted upon by α –amylase & pectinase enzyme

Organism	Pre Treatment	Reducing Sugar after pretreatment (mg/dl)		Reducing sugar after enzyme addition (mg/dl)		Reducing sugar after culture addition (mg/dl)		Reducing sugar Final (mg/dl)	
		α-Amylase Enzyme	Pectinase Enzyme	α-Amylase Enzyme	Pectinase Enzyme	α-Amylase Enzyme	Pectinase Enzyme	α-Amylase Enzyme	Pectinase Enzyme
Control	NaOH	0.21	0.21	0.24	0.24	0.21	0.22	0.28	0.28
SC 25°C		3.00	3.08	0.96	2.28	0.51	2.32	0.20	1.05
ZM 25°C		0.85	3.00	0.70	3.62	0.98	3.21	0.71	0.56
KM 25°C		3.02	3.09	2.90	3.07	2.55	2.98	1.89	1.05
KM 50°C		3.00	3.05	6.66	3.90	2.11	2.05	0.50	0.94

TABLE 2: Percentage of Ethanol produced in Sugarcane bagasse at various stages of process

Organism	Pre Treatment	After pretreatment (%)		After culture addition (%)		Final (%)	
		α -Amylase Enzyme	Pectinase Enzyme	α -Amylase Enzyme	Pectinase Enzyme	α -Amylase Enzyme	Pectinase Enzyme
Control	NaOH	2.00	2.00	2.89	2.89	2.91	2.91
SC 25°C		3.45	3.10	7.93	5.74	12.36	7.59
ZM 25°C		2.43	2.04	8.39	8.39	9.14	9.02
KM 25°C		2.63	2.41	8.39	5.46	10.02	8.39
KM 50°C		3.66	2.29	11.49	5.74	14.66	8.56

TABLE 3: Reducing Sugar content in Ragi husk acted upon by α –Amylase & Pectinase Enzyme

Organism	Pre Treatment	Reducing Sugar after pretreatment (mg/dl)		Reducing sugar after enzyme addition (mg/dl)		Reducing sugar after culture addition (mg/dl)		Reducing sugar Final (mg/dl)	
		α-amylase enzyme	Pectinase enzyme	α-amylase enzyme	Pectinase enzyme	α-amylase enzyme	Pectinase enzyme	α-amylase enzyme	Pectinase enzyme
Control	NaOH	0.67	0.67	0.69	0.69	0.88	0.88	0.72	0.72
Sc 25°c		1.63	1.80	1.73	1.23	1.01	1.09	0.40	0.98
Zm 25°c		1.33	1.33	1.70	1.72	1.63	1.61	1.59	1.03
Km 25°c		1.34	1.41	1.62	1.73	1.11	0.98	0.78	0.94
Km 50°c		1.31	1.43	1.89	1.67	1.21	1.43	0.32	0.41

TABLE 4: Percentage of Ethanol produced in Ragi husk at various stages of process

Organism	Pre Treatment	After pretreatment (%)		After culture addition (%)		Final (%)	
		α -amylase enzyme	Pectinase enzyme	α -amylase enzyme	Pectinase enzyme	α -amylase enzyme	Pectinase enzyme
Control	NaOH	1.01	1.01	1.00	1.00	0.99	0.99
SC 25°c		2.82	1.19	3.04	3.79	9.02	15.40
ZM 25°c		2.71	2.06	10.4	8.33	13.30	13.56
KM 25°c		3.73	2.17	8.68	14.08	10.92	14.78
KM 50°c		3.63	2.29	9.94	3.79	14.94	16.44

TABLE 5: Reducing Sugar content in Broad bean peel acted upon By α –Amylase & Pectinase enzyme

Organism	Pre Treatment	Reducing Sugar after pretreatment (mg/dl)		Reducing sugar after enzyme addition (mg/dl)		Reducing sugar after culture addition (mg/dl)		Reducing sugar Final (mg/dl)	
		α-Amylase Enzyme	Pectinase Enzyme	α-Amylase Enzyme	Pectinase Enzyme	α-Amylase Enzyme	Pectinase Enzyme	α-Amylase Enzyme	Pectinase Enzyme
Control	Hot water	1.67	1.67	1.65	1.65	1.99	1.99	1.89	1.89
SC 25°C		2.73	0.63	1.99	1.93	1.67	1.07	1.43	0.87
ZM 25°C		1.01	1.08	1.80	1.12	1.90	1.09	1.50	0.67
KM 25°C		1.02	1.05	1.09	1.74	1.31	1.33	0.85	1.42
KM 50°C		1.90	1.08	1.66	1.43	1.32	1.34	0.95	1.22
SC 25°C	Dilute acid	0.02	0.04	0.06	0.48	0.02	0.72	0.00	0.33
ZM 25°C		0.70	1.73	0.98	1.62	0.99	1.48	0.62	1.08
KM 25°C		0.62	1.56	0.96	1.42	1.05	1.87	0.43	1.44
KM 50°C		0.82	1.42	0.94	1.21	0.98	1.76	0.62	0.95
SC 25°C	NaOH	1.96	1.62	1.82	2.02	1.93	1.69	0.42	1.30
ZM 25°C		1.09	1.50	1.73	1.43	1.63	1.70	1.72	1.53
KM 25°C		1.06	1.67	1.90	1.86	1.45	1.56	0.61	1.48
KM 50°C		1.80	1.43	1.80	1.21	2.00	0.69	1.63	0.42

TABLE 6: Percentage of Ethanol produced in Broad bean peel at various stages of process

Organism	Pre Treatment	After pretreatment (%)		After culture addition (%)		Final (%)	
		α -Amylase Enzyme	Pectinase Enzyme	α -Amylase Enzyme	Pectinase Enzyme	α -Amylase Enzyme	Pectinase Enzyme
Control	Hot water	2.00	2.00	1.99	1.99	2.04	2.04
SC 25°C		1.14	3.33	4.48	5.46	5.74	6.44
ZM 25°C		2.70	2.58	3.62	3.45	8.45	6.38
KM 25°C		1.72	1.09	2.29	3.73	7.12	3.95

KM 50°C		2.58	3.16	3.45	4.88	7.03	5.84
SC 25°C	Dilute acid	1.55	2.29	2.29	4.77	6.66	7.34
ZM 25°C		1.14	2.18	3.96	5.92	7.47	7.76
KM 25°C		1.49	0.97	3.16	6.15	7.14	6.90
KM 50°C		1.66	1.03	5.28	5.46	6.03	6.43
SC 25°C	Alkali	2.29	2.41	3.45	3.79	6.85	5.73
ZM 25°C		2.70	2.64	3.79	3.45	8.02	5.77
KM 25°C		2.87	3.41	3.96	4.02	7.01	5.15
KM 50°C		1.68	2.87	4.94	4.54	6.61	6.66