DIRECT BIOCONVERSION OF OIL PALM EMPTY FRUIT BUNCHES FOR BIOETHANOL PRODUCTION BY SOLID STATE BIOCONVERSION

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Abstract: Bioethanol production was conducted by utilizing empty fruit bunches (EFB) with the treatment of mixed culture of fungi and yeast in solid state bioconversion process. The compatibility of several fungal and yeast was tested to develop direct solid state bioconversion using the potential mixed culture. The mixed culture of a fungus (*Trichoderma harzianum)* and a yeast (*Saccharomyces cerevisiae)* showed a good potential as they produced higher ethanol (14%) among the other mixed culture combination in liquid-broth fermentation. The optimization of process condition was performed with varied level of moisture content, pH, inoculum size, concentration of co-substrate and mineral solution by using the central composite design (CCD). The optimum process conditions giving maximum ethanol production (14.1%) were: moisture of 60%, pH of 7, inoculum size of 4%, co-substrate concentration of 2% and minerals concentration of 2%. The results indicated that pH, co-substrate and minerals concentration were highly significant ($p<0.01$) followed by moisture content ($p<0.05$) and inoculum size ($p<0.05$). The coefficient of determination (R^2) was 99.6% which satisfied the adjustment of experimental data in the model. A validation experiment was carried out to evaluate the process conditions obtained from the model.

Keywords: *Bioethanol, solid-state bioconversion, empty fruit bunches, central composite design, agricultural solid waste*

1. INTRODUCTION

Ethanol is one of the modern forms of renewable biomass energy produced via sugar fermentation and can be a potential source of sustainable transportation fuel [1]. It has been proposed that an alternative feedstock for biofuel is wasted crops, which is defined as crop lost in distribution [2], replacing the traditional starch crops and can avoid conflicts between human food use and industrial use of crops. Carbohydrates in the wasted crops, like cellulose and hemicellulose are the main potential feedstocks for producing bioethanol whereas lignin can be used to generate electricity or steam [2]. Hence, the use of agricultural residues (straws, hulls, stems, stalks, etc..) are ideally inexpensive and abundantly available for value added applications.

Malaysia is one of the countries in Asia that practiced agriculture as one of its major industries of economic importance. The oil palm industry produces about 90 million

tonnes of lignocellulosic biomass i.e. oil palm biomass (OPB) each year of which about 40 million tonnes are the empty fruit bunches (EFB), oil palm trunks (OPT) and oil palm fronds (OPF) [3]. OPB is often left in the plantation to provide organic nutrients to the trees or burned illegally [4]. At the crude palm oil mills, fibers and shells from oil palm biomass are also used as boiler fuel to produce process steam for sterilization and also possibly for electricity generation [5].

A number of processes and the action of various microorganisms have been studied to produce ethanol from cellulosic materials. Among them, a multistage approach for ethanol production from cellulosic substances has been speculated to be the most applicable for the industrial scale ethanol production [6]. This process involves pretreatment of agroresidues [7], saccharification of cellulosic materials by the mixed cellulolytic enzymes [8], production of sugars and fermentation of sugars to ethanol [9]. The disadvantages of these processes are involvement of varieties of unit operation, and an energy-intensive system that increase the production cost [6]. Other available approaches are simultaneous saccharification of agricultural residues and fermentation into ethanol [10]. The direct bioconversion of cellulosic materials by *Clostridium thermocellum* is not attractive because of low ethanol productivity and high energy requirements compared to ethanol production by *S. cerevisiae* using molasses [11]. A suitable alternative process for the development of compatible mixed culture of lignocellulolytic fungi and yeast is needed that might increase the yield of ethanol production. The present work was undertaken to develop the potential compatible mixed culture of fungi and yeast for reasonably high production of ethanol through optimum process conditions by utilizing EFB as the raw material.

2. MATERIALS AND METHODS

2.1 Fermentation Media and Culture

The substrate used for this study was empty fruit bunches (EFB) because they are cheap source of lignocellulose and readily available. The EFB was obtained from Seri Ulu Langat Palm Oil Mill, Dengkil, Selangor, Malaysia. The EFB sample was stored at 4 °C until further use. Four fungal strains and one yeast strain were used and all of them were obtained from laboratory stock. Lignocellulosic materials decomposers such as *Phanerocheate chrysosporium, Trichoderma harzianum*, *Mucor hiemalis* and *Aspergillus niger (SS-103)* were maintained on potato dextrose agar (PDA) plates for spore production and incubated at 37 °C while *S. cerevisiae* was maintained on yeast extract-malt extract-peptone-glucose (YMPG, Difco).

2.2 Inoculum and Subculture Preparation

The four fungal strains were prepared in using standard protocol described here, except for *S. cerevisiae* (yeast). Fungal strains were cultured on 3.9% potato dextrose agar (PDA) as inocula sources [12]. The fungal strains inocula were prepared by washing the seven days growth culture on PDA plate with 100 ml sterile distilled water. The spore suspensions were filtered (Whatman no.1) and stored in ~4 °C refrigerator until further

use. Yeast inoculum was prepared by transferring two loops of cells from YMPG media (2% malt extract and 1% yeast extract) which was incubated at 25 °C for 24 hours. *S. cerevisiae* can be sub-cultured on PDA plate and stored for only 14 days of its shelf life.

2.3 Compatibility Test of Mixed Cultures

The compatibility test was done to evaluate the direct (single-step) bioconversion for bioethanol production using different fungi and a yeast. As yeast is the ethanol producer microbe, the compatibility mixed culture test involved the yeast with other lignocellulose degrading fungi: *P. chrysosporium* (PC) and *S. cerevisiae* (SC); *T. harzanium* (TH) and *S. cerevisiae* (SC); *M. hiemalis* (MH) and *S. cerevisiae* (SC); *A. niger 103* (AN) and *S. cerevisiae* (SC).

The evaluation of compatible mixed culture was studied in liquid and solid media (2% malt extract) by observing the mutual growth in plates for 6 days and bioethanol production in liquid cultures for 2 days at 30 °C.

2.4 Treatment of Substrate

Treatment of the substrate (EFB) is based on the method suggested by Ghosh and Deb [13]. The EFB was washed thoroughly to make them dust free and then dried. Dried EFB were crushed into smaller particles in the range of 100 - 500 μm in size by using a grinding machine.

2.5 Development of Direct Bioconversion Process with Compatible Mixed Culture

Experimens were carried out in 250 ml Erlenmeyer flasks for the development of the direct bioconversion process with compatible mixed culture. Four runs were conducted to evaluate the process with potential mixed culture of a fungus and a yeast. Three types of fungi were used; *T. harzianum*, *P. chrysosporium* and *M. hiemalis* while the yeast used was *S. cerevisiae*. Sample (20 g) for every run consisted of 28% substrate (EFB), 2% co-substrate (wheat flour), 59% sterile distilled water, 5% minerals solution and 6% inoculum which was equally distributed depending on how many types of microbes were used (Alam *et al.* 2005). The experimental design is shown in Table 1.

Table 1: Development of direct bioconversion with different fungi and yeast and incubation time.

Run	Combination	Incubation time	Sampling time
1	*T. harzanium* and *S. cerevisiae* (TH-SC)	Both strains at the same time	2, 4, 6, 7, 8
2	*T. harzanium* and *S. cerevisiae* (TH-SC)	TH was inoculated at the beginning and SC on the 5th day of run	2, 4, 6, 7, 8
3	*P. chrysosporium, T. harzanium* and *S. cerevisiae*	- PC was inoculated at the beginning, TH on the 4th	2, 4, 6, 7, 8

	(PC-TH-SC)	day and SC on the 6th day of run	
4	*M. hiemalis* and *S. cerevisiae* (MH-SC)	MH was inoculated at the beginning and SC on the 5th day of run	2, 4, 6, 7, 8

2.6 Experimental Design and Optimization of Process Conditions for Bioethanol Production

Experimental design for optimization of process conditions was done using central composite design (CCD). Five factors were considered for optimization: moisture content, pH, inoculum size, co-substrate and minerals concentration. The range of these parameter were analyzed in the Minitab software which is capable of generating the number of experiments with different formulation in order to screen the best optimum value of each parameter for ethanol production. Three replicates with five levels of CCD were applied. The different levels given in codes and the actual values for CCD are shown in Table 2.

Table 2: Level of process conditions as factors for central composite design (CCD).

Component	Unit	Level				
		-2	-1	0	1	2
Moisture	%(v/w)	50	60	70	80	90
pH	-	4	5	6	7	8
Inoculum	%(v/v)	2	4	6	8	10
Co-substrate	%(v/v)	1	2	3	4	5
Minerals	%(v/v)	1	2	3	4	5

A second order polynomial equation was developed using the experimental results according to the CCD through multiple regression analysis. For a five-factor system, the model equation is as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{55} X_5^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{15} X_1 X_5 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{25} X_2 X_5 + \beta_{34} X_3 X_4 + \beta_{35} X_3 X_5 + \beta_{45} X_4 X_5 \quad (1)$$

where Y is the bioethanol production (% v/v), predicted response; β_0, intercept β_1, β_2, β_3, β_4, β_5, linear coefficients; β_{11}, β_{22}, β_{33}, β_{44}, β_{55}, squared coefficient: β_{12}, β_{13}, β_{14}, β_{15}, β_{23}, β_{24}, β_{25}, β_{34}, β_{35}, β_{45}, interaction coefficients.

2.7 Analytical and Statistical Analysis

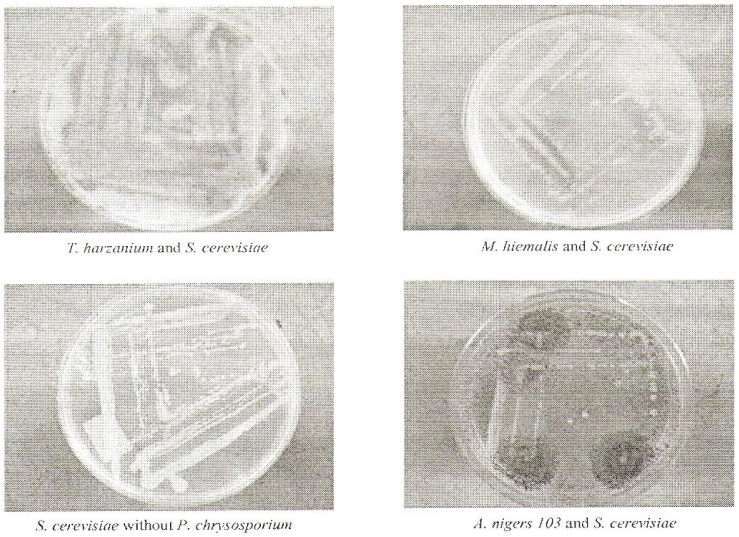
Samples (in duplicates) were withdrawn from the incubator after 2, 4, 6, 7 and 8 days. The fermented substrate was treated with 30 ml distilled water and shaken for 2 hours at room temperature (30 ± 2^0C) and the liquid extract was filtered using Whatman no. 1 filter paper and collected. The liquid extract was used for ethanol estimation [14] and reducing sugar analysis [15]. Several techniques such as ANOVA, t-test, p-values were utilized to evaluate the model as well as for the optimization process.

3. RESULTS AND DISCUSSION

3.1 Compatibility Test of Fungal Mixed Cultures and Yeast

In the liquid culture, each combination of mixed culture showed growth which was indicated by the change in color and turbidity. The PC-SC combination showed that *S. cerevisiae* inhibited the growth of *P. Chrysosporium,* the latter which usually grows in 'pellet-shaped' in the shake flask (data not shown).

After 6 days, each combination of mixed culture was cultured on PDA plates for another six days to observe the compatibility effects. The visual observation of the compatibility test is shown in Fig. 1. Out of the four combinations, three mixed culture showed compatibility with each other: TH-SC, MH-SC and AN-SC combinations. For PC-SC combination, *P. chrysosporium* did not grow well in liquid media and on PDA plate.



T. harzanium and *S. cerevisiae* | *M. hiemalis* and *S. cerevisiae*

S. cerevisiae without *P. chrysosporium* | *A. nigers 103* and *S. cerevisiae*

Fig. 1: Compatibility of mixed culture on PDA plates.

The production of ethanol after 6 days of fermentation is shown in Fig. 2. The results indicated that the highest ethanol production (14.2%, v/v) was observed by TH-SC combination whereas the lowest was 6.4 % (v/v) in AN-SC combination. From the observation and estimation of ethanol yield, *T. harzanium* and *S. cerevisiae* were mutually grown together in liquid and solid media and proved to be an excellent choice for ethanol production compared to the other combinations.

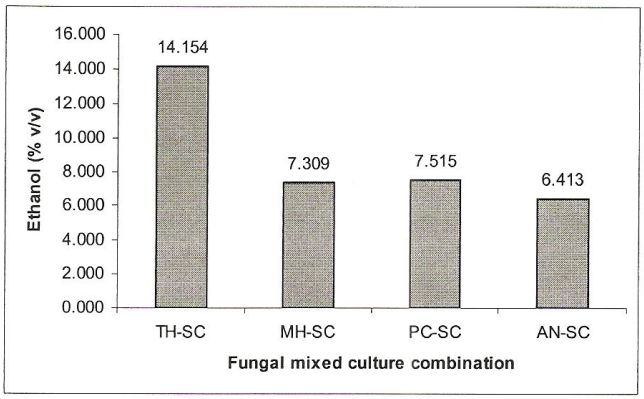


Fig. 2: Production of ethanol (% v/v) with different mixed culture.

3.2 Development of Direct Bioconversion Process with Compatible Mixed Culture

3.2.1. Production of Bioethanol

The development of a direct bioconversion (single-step) of empty fruit bunches with compatible mixed culture was carried out. Four experimental runs were conducted with varying inoculation time in the presence of the mixed culture. The concentration of ethanol increased with fermentation time (Fig. 3). Run 1 which contained *T. harzianum* and *S. cerevisiae* mixed culture showed the best ethanol production compared to other experimental runs.

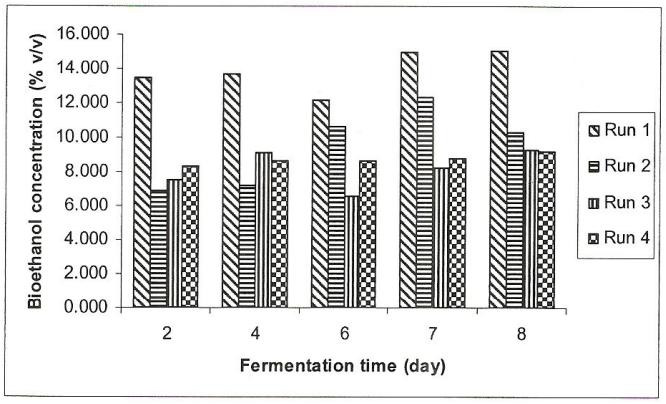


Fig. 3: Production of ethanol (% v/v) for different experimental runs with fermentation time.

The highest amount of ethanol produced recorded on the last day of fermentation for Run 1, was 15.1 % (v/v). Ethanol concentration of 15.0 % (v/v) was obtained in 72 hours fermentation of Jerusalem Artichoke Tubers in mixed culture of *S. cerevisiae* and *A. niger* [17]. The trend of ethanol production observed in the present study was as predicted where ethanol production increases throughout the fermentation days. The slight decline of ethanol production on the sixth day of fermentation before slightly increased towards the end of fermentation as observed in Run 1 and Run 3 perhaps can be explained that the ethanol is a carbon source that might be utilized by the microorganisms as food for their metabolism during the fermentation.

3.2.2. Determination of Reducing Sugar

As shown in Fig. 4, the experimental run 4 showed higher content of reducing sugar compared to the other runs. The strain *M. hiemalis* has been proven to hydrolyze lignocellulose of EFB to reducing sugar more efficient than the *T. harzanium* and *P. chrysosporium*. The highest reducing sugar concentration for Run 4 (0.44 g/L) was recorded on the second day of fermentation. The concentration of reducing sugar decreased during the fermentation coinciding with the increase of biomass and ethanol production [18]. This trend is shown by all runs except for Run 1 where the concentration of reducing sugar decreases rapidly. Roukas [18] also found that the concentration of reducing sugar decreased rapidly during the 24 hours fermentation with only *S. cerevisiae* used in carob pods. The reducing sugar concentration for Run 4 is highest in in all sampling times compared to the other runs. The lowest concentration of reducing sugar was 0.22 g/L for Run 3 in both 6 and 7 days of fermentation time.

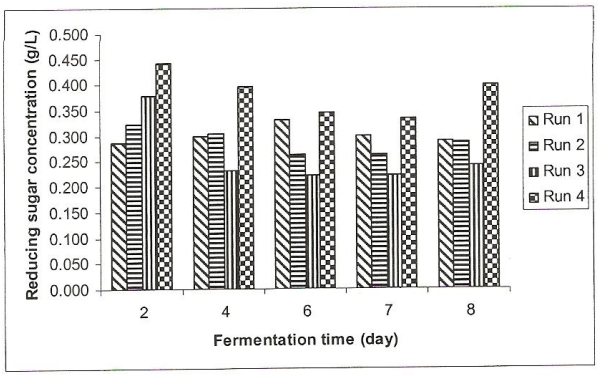


Fig. 4: Reducing sugar concentration (g/l) during bioconversion of EFB into ethanol production.

3.3 Optimization of Process Conditions for Bioethanol Production

In order to determine the optimum process conditions, a total of 27 treatments for optimization were determined by using a statistical software applying CCD with different

range of the parameters. The polynomial regression model relating the production of bioethanol with independent variables X_1, X_2, X_3, X4 and X_5 are as follows:

$$Y = 130 - 0.649X_1 - 19.6X_2 - 2.78X_3 - 8.24X_4 - 10.3X_5 + 0.000065X_1X_1 + 1.51X_2X_2 + 0.303X_3X_3 + 0.850X_4X_4 + 1.28X_5X_5 + 0.0440X_1X_2 + 0.0157X_1X_3 - 0.00073X_1X_4 + 0.0369X_1X_5 - 0.396X_2X_3 + 0.451X_2X_4 - 0.145X_2X_5 + 0.0342X_3X_4 + 0.0983X_3X_5 + 0.0547X_4X_5 \quad (2)$$

where Y is the bioethanol production (% v/v), and X_1, X_2, X_3, X_4, X_5 are the independent variables; moisture content, pH, inoculum size, co-substrate and mineral solution respectively. The observed (experimental) values along with the predicted values of bioethanol are shown in Table 3. The process conditions in Run 9 resulted to a maximum bioethanol production of 14.1 % (v/v) although it was slightly less than the predicted ethanol.

Table 3: Experimental and predicted values of total ethanol using various combinations of medium constituents.

Run	Moisture (%v/w) X_1	pH X_2	Inoculum (%v/v) X_3	Co-substrate (%v/v) X_4	Mineral (%v/v) X_5	Ethanol concentration (% by volume)	
						Observed	Predicted
1	70	6	6	3	5	11.041	11.039
2	60	7	8	2	4	10.270	10.429
3	90	6	6	3	3	2.887	2.788
4	60	7	8	4	2	12.006	12.192
5	80	5	8	4	2	8.287	8.711
6	70	6	6	3	3	5.752	6.225
7	50	6	6	3	3	9.470	9.715
8	60	5	8	2	2	13.879	14.271
9	60	7	4	2	2	14.127	14.315
10	80	7	8	2	2	8.479	8.800
11	70	6	10	3	3	11.051	10.980
12	60	5	4	2	4	12.419	12.682
13	60	5	4	4	2	12.281	12.574
14	60	5	8	4	4	12.667	12.928
15	70	4	6	3	3	12.061	11.982
16	70	6	6	5	3	9.223	9.394
17	80	5	8	2	4	10.441	10.838
18	80	5	4	4	4	6.992	7.288
19	80	7	8	4	4	9.939	10.128
20	70	6	6	1	3	9.884	9.857
21	70	8	6	3	3	12.364	12.550
22	60	7	4	4	4	13.080	13.136
23	70	6	6	3	1	11.510	11.652
24	70	6	2	3	3	10.959	11.168
25	80	5	4	2	2	7.818	8.244
26	80	7	4	4	2	10.573	10.790

27	80	7	4	2	4	9.829	10.019

From the statistical software, the regression equation and determination coefficient R^2 were evaluated to test the fit of the design of experiment or model. The model resulted in a high determination coefficient R^2 of 0.996 which means 99.6 % of the factors; moisture content, pH, inoculum size, co-substrate concentration and mineral solution in the response or correlated with each other.

The value of the adjusted determination coefficient was also shown to be very high (98.4%) which suggests the high significance of the model [19, 20]. The corresponding analysis of variance (ANOVA) is presented in Table 4. The ANOVA of quadratic regression model demonstrates the model is highly significant, as this model showed very low probability value which is $p_{model} > F = 0.000$

Table 4: ANOVA for the selected quadratic model.

Source	Degree of freedom	Sum of Squares	Mean Squares	F-value	p>F
Regression	20	164.3988	82.68	82.68	0.000
Residual Error	6	0.5965	0.0994		
Total	26	164.9953			

The t-distribution and the corresponding p-values of the variable estimation were evaluated using Minitab software. The significance of each coefficient or factor was determined by t-values and p-values. The pattern of interactions between the variables is indicated by these coefficients. The variables with low probability levels contribute to the model, whereas others with high probability level can be neglected and eliminated from the model. t-value with larger magnitude and smaller p-value indicated the high significance of the corresponding coefficient or factor [21]. The t-values and p-values for the linear, quadratic and the iterative terms are presented in Table 5.

From the statistical analysis, it can be evaluated that the variable with the largest effect was the squared term of pH (X_2X_2), inoculum size (X_3X_3), co-substrate (X_4X_4) and minerals solution (X_5X_5). Furthermore, the linear term of moisture (X_1), pH (X_2), inoculum size (X_3), co-substrate (X_4) and minerals (X_5) and also the interaction between moisture and pH (X_1X_2); moisture and inoculum size (X_1X_3); moisture and minerals solution (X_1X_5); pH and inoculum size (X_2X_3); pH and co-substrate (X_2X_4) and inoculum size with minerals solution (X_3X_5) were more significant than the other factors.

In most studies on solid state fermentation of ethanol production, moisture level is reported to be higher in producing high amount of product. The highest values of fermentation parameters were achieved at a moisture level of 70% [18]. It is reported that most of the condition in ethanol production is acidic since the yeast was able to grow and efficiently ferment ethanol at pH of 3.5 to 6.0 in temperature of 28 °C to 35 °C [22].

The increase of inoculum size would increase the biomass concentration in the fermentation. A study by Roukas [18] has reported that the maximal ethanol concentration (160 ± 3 g/kg dry pods), were obtained with an initial inoculum of 3% (5.0 x 10^8 cell/g carob pulps). He also reported that larger initial inoculum size of 12% resulted in maximum biomass concentration but lower ethanol concentration. Maximum production

rate of ethanol was observed at low levels of $NH_4H_2PO_4$ as minerals in the fermentation [23].

Table 5: Statistical analysis showing coefficient of t-value and p-value.

Predictor	Coefficient	Standard error coefficient	t-value	p-value
Constant	129.675	8.647	15.00	0.000
Moisture, X_1	-0.6490	0.1270	-5.11	0.002**
pH, X_2	-19.632	1.171	-16.77	0.000**
Inoculum, X_3	-2.7780	0.4658	-5.96	0.001**
Co-substrate, X_4	-8.2445	0.9316	-8.85	0.000**
Minerals, X_5	-10.2650	0.9316	-11.02	0.000**
X_1X_1	0.0000652	0.0007883	0.08	0.937
X_2X_2	1.51491	0.07883	19.22	0.000**
X_3X_3	0.30327	0.01971	15.39	0.000**
X_4X_4	0.85031	0.07883	10.79	0.000**
X_5X_5	1.28075	0.07883	16.25	0.000**
X_1X_2	0.044039	0.007883	5.59	0.001**
X_1X_3	0.015687	0.003941	3.98	0.007**
X_1X_4	-0.000727	0.007883	-0.09	0.930
X_1X_5	0.036883	0.007883	4.68	0.003**
X_2X_3	-0.39620	0.03941	-10.05	0.000**
X_2X_4	0.45148	0.07883	5.73	0.001**
X_2X_5	-0.14499	0.7883	-1.84	0.115
X_3X_4	0.03425	0.03941	0.87	0.418
X_3X_5	0.09833	0.03941	2.49	0.047*
X_4X_5	0.05472	0.07883	0.69	0.514

**$p<0.01$; *$p<0.05$

An experiment was conducted to validate the model, and determine the maximum production of ethanol with optimum process conditions which were moisture of 60% (v/w); pH of 7; inoculum size of 4% (v/v); co-substrate of 2% (v/v) and minerals solution of 2% (v/v). As shown in Fig. 5, the highest production of ethanol was 13.1% (v/v) on the first day of fermentation. The result revealed that the bioethanol production from the experiment (13% v/v) validated the model which showed the bioethanol production of about 14%, slightly higher on the first day of treatment in single-step of solid state bioconversion using compatible mixed culture.

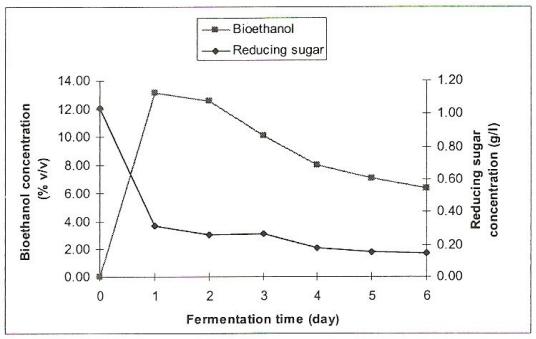


Figure 5: Bioethanol concentration (% v/v) and reducing sugar concentration (g/l) versus fermentation time (day).

4. CONCLUSION

The results from the present work lead to a conclusion that the process factor had a profound effect on the ethanol production using developed bioconversion process. Among the experimental runs, Run 9 with 60% (v/w) moisture content, pH 7, 4% (v/v) inoculum size, 2% (v/v) co-substrate and 2% (v/v) minerals solution showed the highest production of ethanol (14.1%) and its prediction was 14.3% (v/v). The study showed that the production of bioethanol from EFB by direct solid-state bioconversion could be an attractive candidate for a better solid waste management through safe and environmental friendly disposal.

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