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Rice Straw from Saccharification to Ethanol Simultaneous Fermentation using *Aspergillus neoellipticus* and *Saccharomyces cerevisiae*

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ABSTRACT

One fungal isolate Am1 from compost identified as *Aspergillus neoellipticus* [GenBank accessed no. OM760561] was found to possess cellulytic activity of 5806 U (Fpase) when grown in submerged culture using treated rice straw (RS) as substrate. Under successive optimization steps including pH, nitrogen source type and C:N ratio, cellulytic activity increased by 1.4-fold recording 8237 U utilizing yeast extract at pH 6 and C:N ratio of 1:0.9. Saccharification process using lyophilized raw cellulases produced by *A. neoellipticus* was optimized achieving saccharification efficiency of 89% at 50ºC and pH7. In a simultaneous saccharification fermentation process using 2L fermentor, optimized saccharification of RS was first held at an enzyme load of 25 U/ g RS after which *Saccharomyces cerevisiae* was used to ferment the resulting reducing sugar at a concentration of 10%. Optimized fermentation achieved fermentation efficiency of 99.2% at pH6 after 48 h, at a yield of 0.6g ethanol/g reducing sugar or 37.5 g ethanol/ 100 g RS.

Keywords: Ethanol, Rice Straw, Cellulase, Saccharification, Fermentation, *Aspergillus neoellipticus*.

1. Introduction

Agricultural residues in Egypt were evaluated for their availability in many studies, of which rice cultivation area of 0.55 million hectare [Mha] formed straw byproduct straw a total of 1.79 million ton/year [Mt/year] after excluding over 75% included in soil conservation, agro-industrial applications, losses in handling, transportation and storage (Abdelhady *et al.,* 2021).

The potential use of rice straw for energy production in Egypt is an essential prospect sharing in reduction of harmful greenhouse gases (SO_2, NO_2, CH_4, N_2O, CO) resulting from open burning (Datroit *et al.,* 2007). In one field of energy, Abdelhady *et al.,* (2014) investigated power plant fired with rice straw assumed to provide annual electric energy. Another major field that has been contributing biofuels production from carbon sources in which bioethanol production and development studies predominated biobutanol, biodiesel, biogas and biohydrogen (Zabed *et al.,* 2014).

The availability of carbon content in rice straw is embedded in its cellulosic structure after being exposed from lignin contents through variable pretreatments to enhance cellulose digestion (Rabeya *et al.,* 2020).

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Jiao *et al.,* (2021) pointed out for the wide share of fungal cellulase production applied in cellulose degradation. Many attempts to produce cellulase by several types of fungal in submerged culture using agricultural residues were surveyed, including *A. fumigatus* (Stewart and Parry, 1981; Kalogeris *et al.,* 2003), *A. flavus* (Kumar *et al.,* 2020), *A. niger (*Awodi *et al.,* 2021; Noguchi *et al.,* 2021), *Fusarium oxysporum* (Li *et al.,* 2011) and *Trichoderma reesei* (Kumar *et al.,* 2018; Abouzied *et al.,* 1986).

Many studies attempted to gather processes of efficient saccharification of pretreated rice by the aid of fungal cellulase in the same bioreactor with submerged fermentation of the released reducing sugar by yeasts including *Saccharomyces cerevisiae* (Rabeya *et al.,* 2021; Li *et al.,* 2011; Ismail and Hassan, 2020) and *Candida tropicalis* (Shuler and Kargi, 1992), in what so called simultaneous saccharification and fermentation [SSF] under optimized conditions.

The present work several optimization studies aimed to maximize cellulase production by fungal isolate through submerged culture, followed by simultaneous saccharification of pretreated rice straw and fermentation of released reducing sugar in submerged batch fermentation process in which correlation studies between interacting measurable parameters were used to clarify their results.

2. Materials and Methods

2.1. Isolation of cellulytic fungi

Mineral salt agar medium (MSM) in plates was used for isolation, according to Mandels and Weber (1969), modified by adding carboxy methyl cellulose (CMC) 1%. The sample from compost pile was serially diluted and transferred onto the MSM agar, where the fungal colony surrounded with the largest clear zone was chosen and purified as the seldom cellulytic isolate for further testing. The fungal isolate was maintained regularly by subculturing on PDA slants.

2.2. Rice straw

Rice straw purchased from local farm was roughly cut, washed in tap water and dried under vacuum at 60°C. Rice straw was then delignified using alkaline/hypochlorite pretreatment according to Wise *et al.,* (1946) Lignin and holocellulose contents were analyzed before and after delignification as described by Sluiter *et al.,* (2008).

2.3. Cellulase production

Fungal isolate was grown on PDA slants at 35ºC/24hr, after which the conidia were harvested by flooding in sterile 0.85% saline and its concentration was adjusted at $1\text{-}5x10^{-5}$ cfu/ml using a hemocytometer, as described by Lass-Flörl *et al.,* (2003). The hyphae suspension was applied for cellulase production medium at concentration of 10% (v/v) in 100ml MSM broth amended with 1% delignified rice straw (RS) as stated by Sarkar and Aikat (2014) within conical flask (250ml), while pH was adjusted at 5 and incubation carried out at 37°C on shaker incubator (125rpm).

The cellulase production by the fungal isolate was subjected to successive optimization studies adjusted individually to reveal the most suitable initial pH value, nitrogen source and C:N ratio, targeting maximum yield regardless to period needed. Buffers used were sodium acetate (0.1M) for pH 4 and 5, while Potassium phosphate (0.1M) for pH 6 and 7. Nitrogen sources used included $(NH_4)_2SO_4$, NH_4NO_3, yeast extract and beef extract in quantities equivalent to nitrogen content of 0.6g /L. Under the optimized conditions, the fungal isolate was provoked to produce cellulase conducted in 500ml /1L conical flask under standard incubation conditions (37°C/150rpm) and the resulting supernatant considered as raw cellulase was lyophilized to powder form.

2.4. Saccharification

The lyophilized cellulase was dissolved in buffer of desired pH value (5, 6 and 7) and assayed. The volume containing cellulytic activity of 25U was mixed with 5 ml buffer of same pH containing 0.1g RS (2% RS, enzyme load 250 U/g RS) in each test tube. Variable incubation temperatures (40

and 50ºC) were studied in permutations and combinations with pH values (5, 6 and 7) for their effect of on saccharification. At optimum temperature and pH, saccharification was scaled up in bottles with larger volume (70ml) and more enzyme load (25U for 1.4% RS, enzyme load 25.5U/g RS).

2.5. Cellulytic activity assay

Cellulolytic enzyme activity assayed as FPase according to Ghose (1987). The reaction mixture contained 1ml of supernatant from production culture broth (crude enzyme) or enzyme solution (in buffer) and 50 mg/ Whatman No 1 filter paper per 1ml 0.05M citrate buffer pH 4.8 incubated at 50°C for 60 min. The reaction was stopped by the addition of 3ml DNS reagent for determination of the released reducing sugars as glucose.

Cellulytic activity unit (U) was defined as the amount of enzyme required for liberating 1µg of reducing sugars as glucose in 1ml/min or 1g/min which is equivalent to 180 µmole of reducing sugars as glucose in 1ml/min (IU) stated by Sarkar and Aikat (2014).

2.6. Reducing sugars assay

Reducing sugars content was determined according to the method of Miller *et al.,* (1959) using 3, 5-dinitrosalsilic acid (DNS) reagent and the absorbance was measured at 640nm using Perkin-Elmer 55E spectrophotometer.

2.7. Fermentation

2.7.1. Fermentation Medium

Fermentation medium used as mentioned by Taylor *et al.,* (1995) contained (g/L) Yeast extract (8.5), NH_4Cl (1.3), Ca Cl_2 (0.06), $MgSO_4$.7H2O (0.12), while supplemented with saccharified RS whose glucose content nearly equivalent to 100g glucose as carbon source.

2.7.2. Fermentation Bottle

Saccharified RS was transferred into 200 ml narrow neck glass bottles supplemented with the sterile fermentation media in 180 ml total media volume, firmly and aseptically stoppered. The initial pH values were adjusted at 4, 5 and 6 individually under aseptic conditions to test their effect on sugar fermentation efficiency % by yeast at 35°C.

The yeast *S. cerevisiae* (Baker's yeast) packed in dry form was purchased from local market to be used as inoculum adjusted at 8g/L (w/v) dry cells in fermentation medium, as indicated by Maria *et al.,* (2014). Fermentation was carried out for 48 hours in triplicates where samples were taken first after 1 hour then every 3 hours as possible.

2.7.3. Fermentor

Scaling up to a 2 L fermentor was dependent on optimized parameter results from fermentation bottle test. The 2L fermentor was first adjusted to carry out saccharification of 30g treated RS using the lyophilized cellulases was mixed in 1.5L buffer 0.5M of suitable pH value. The enzyme load (U/g RS) calculated depending on saccharification test was used and incubated at 50°C and with agitation of 100 rpm.

The process had been carried out for a period to release enough sugar, that after wards when amended with sterile fermentation media components its final volume was increased up to 1.8L so that it contained a final reducing sugar concentration of 10 %. Yeast inoculum was adjusted as dry cells at 8g/L of fermentation medium. The initial pH was adjusted at optimum while incubation at 35°C was carried out with no agitation and no introduced aeration.

2.8. Ethanol assay

Ethanol was determined according to the method described by Caputi *et al.,* (1968) using Potassium dichromate ($K_2Cr_2O_7$) and measured spectrophotometrically at 600 nm.

2.9. Gas chromatography–mass spectrometry analysis (GC-MS)

The SUP from fermentation broth under investigation was kept in freezer until transferred in a cooled state to be analyzed through GC-MS to evaluate the fermentation products including ethanol, CO2 and any possible dissolved oxygen. The GC-MS system (Agilent Technologies) was equipped with gas chromatograph (7890B) and mass spectrometer detector (5977A) at Central Laboratories Network, National Research Centre, Cairo, Egypt.

The GC was equipped with DB-624 column (30 m x 0.32 mm internal diameter and 1.8 μm film thickness). Analyses were carried out using helium as the carrier gas at a flow rate of 1.0 ml/min at a split less, injection volume of 1 µl and the following temperature program: 35 °C for 5 min; rising at 10 °C /min to 70 °C and held for 4.3 min. The injector and detector were held at 90 °C and 260 °C, respectively.

Mass spectra were obtained by electron ionization (EI) at 70 eV; using a spectral range of m/z 50-550. The mass temperature was 230°C and Quad 150 °C. Identification of different constituents was determined by comparing the spectrum fragmentation pattern with those stored in Wiley and NIST Mass Spectral Library data.

2.10. Molecular identification of fungal isolate

The fungal strain was cultivated on Czapek`s yeast extract agar (CYA) medium at 28°C for 5 days. The culture was sent to the Molecular Biology Research Unit, Assiut University for DNA extraction.

2.10.1. DNA extraction, PCR and sequencing of ITS

The DNA extraction was performed following the method of Moubasher *et al.,* (2019). In which, a small portion from fungal growth of 3-day-old culture of *Aspergillus* sp. isolate Am1 grown on malt extract agar (MEA) at 25 °C, were collected and transferred to 2 ml-Eppendorf tube. PCR was conducted according to CTAB method described by Al-Bedak and Moubasher (2020). The universal primers ITS1 and ITS4 were used for amplification of the internal transcribed spacer (ITS) region (White *et al.,* 1990).

2.10.2. Alignments and phylogenetic analyses

Sequences of *Aspergillus* sp. isolate Am1 in this study were assembled using DNASTAR computer package (version 5.05). Assembled sequence of *Aspergillus* sp. isolate Am1 was uploaded to GenBank as OM760501. The closely similar sequences to *Aspergillus*: section Fumigati including sequences of type and ex-type species were downloaded from GenBank. All sequences in this analysis were aligned together using MAFFT (version 6.861b) with the default option (Katoh and Standley, 2013).

Alignment gaps and parsimony uninformative characters were treated by BMGE. Maximum-likelihood (ML) and Maximum parsimony (MP) phylogenetic analyses were performed using PhyML 3.0 (Guindon *et al.,* 2010).

The robustness of the most parsimonious trees was evaluated by 1000 bootstrap replications (Felsenstein *et al.,* 1985) The best optimal model of nucleotide substitution for the ML analyses was determined using Akaike Information Criterion (AIC) as implemented in Modeltest 3.7 (Posada and Grandall, 1998).

The phylogenetic tree was drawn and visualized using MEGA X version 10.2.6 (Kumar *et al.,* 2018). The resulting tree was edited using Microsoft Power Point (2016) and saved as TIF file.

2.11. Calculations

Saccharification percentage was calculated according to Spano *et al.,* (1976):

$$\text{Saccharification \%} = \frac{\text{Total released reducing sugars (mg/g R S)}}{\text{Total carbohydrate in substrate (mg/g RS)}} \times 0.9 \times 100$$

Ethanol yield and fermentation efficiency were calculated according to Hatzis *et al.,* (1996):

$$\text{Ethanol Yield} = \frac{\text{Ethanol produced (g/L)}}{\text{Initial glucose in substrate (g/L)}}$$

$$\text{Fermentation efficiency \%} = \frac{\text{Ethanol actual yield}}{\text{Ethanol theoretical yield}} \times 100$$

Where Ethanol theoretical yield is 51.2%.

2.12. Statistical analysis

All results data were accomplished in triplicates and statistically evaluated by least significant differences (LSD) in one way analysis of variance (ANOVA) at significance of 5% calculated using CoHort software under windows (Costat, model 6.311). The standard deviation (SD) and the standard error (SE) of mean for the experimental measurement triplicates and correlation coefficients were all calculated in Excel work sheet (Microsoft Office 2010).

3. Results

3.1. The genetic identification of the fungal isolate

Phylogenetic analysis of ITS dataset was employed to determine the taxonomic status of our strain relative to other members of *Aspergillus*: section Fumigati. The entire ITS dataset comprised 43 sequences.

The maximum parsimony dataset consisted of 651 characters with 553 constant characters (no gaps, no N), 71 variable characters which were parsimony-uninformative (12.8% of constant characters), and 27 characters were counted as parsimony informative (4.9% of constant). Tamura-Nei (TN93+G+I) was the perfect model for nucleotide substitution.

The dataset for maximum parsimony yielded 2 most parsimonious trees with a tree length of 170 steps, a consistency index is of 0.563107, a retention index of 0.810127, and a composite index of 0.456188, for all sites and parsimony-informative sites. The best scoring ML tree (Figure 1) with the final ML optimization likelihood value of -3442.32 was selected to represent and discuss the phylogenetic relationships among taxa.

The isolate Am 1 occupied the same branch as *Aspergillus neoellipticus* ATCC 16903 (type strain) with 100% (562/562) similarity between both species. As a result, this isolate was identified as *Aspergillus neoellipticus.*

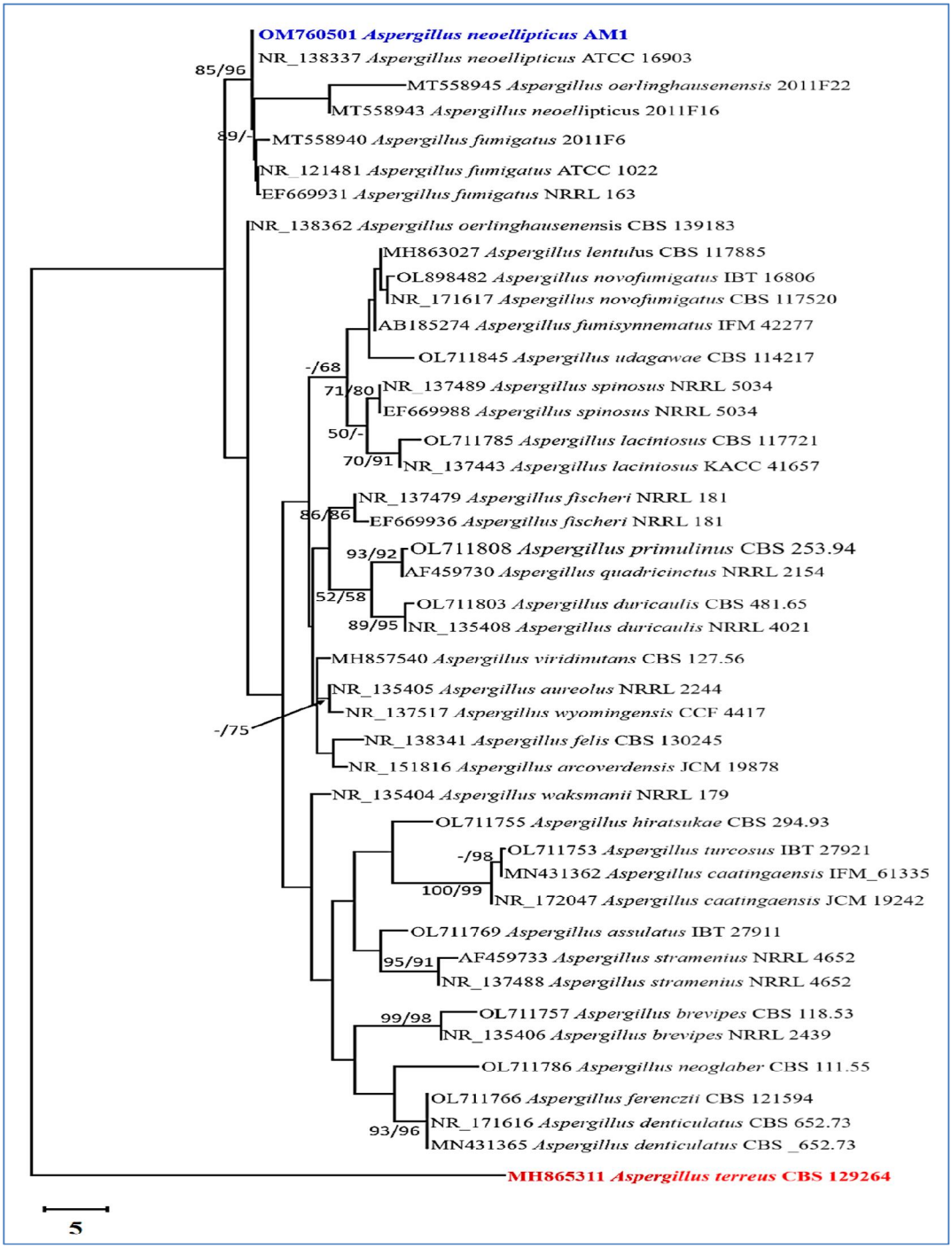

Fig. 1: The first of 1 000 equally most parsimonious trees obtained from a heuristic search (1000 replications) of *Aspergillus neoellipticus* isolate Am 1 (in blue color) compared to other closely similar ITS sequences belonging to genus *Aspergillus*: section Fumigati in GenBank. Bootstrap support values for ML/MP ≥50% are indicated above/below the respective nodes. The nodes lacking bootstrap support (<50%) are marked with (-). The tree is rooted to *Aspergillus terreus* CBS 129264 as outgroup (in red color).

3.2. Delignified rice straw analysis

lignin content in rice straw decreased by 44% due to pretreatment causing loss in weight by 35%, while total carbohydrate calculated as holocellulose increased by 10.6%, calculated from results shown in Table (1).

Table 1: Alterations in weight and main chemical composition in rice straw after pretreatment

Rice straw	Weight loss, %	Main chemical composition, % (w/w)			
		Cellulose	Holocellulose	Lignin	Ash
Native	0	43.93	66.98	14.40	18.62
Treated	35.20	66.82	74.92	8.02	17.06
± %	-44		+10.6	-35	

(± %): Change % either decrease [-] or increase [+]

3.3. Optimizing cellulase production

3.3.1. Optimum pH

Maximum cellulytic production achieved by *A. neoellipticus* after 5 days was 6203U at initial pH 6, according to results illustrated in Figure (1). Initial pH values were adjusted while no buffering agent was added in production media that can maintain pH during incubation period, subsequently variations in pH values during the 9 days of incubation were observed. Drop in pH value was observed mostly among all initial pH values after 3 days of incubation, followed by maximal increase in Fpase activities individually under each initial pH tested.

It was an essential issue to emphasize the impact of incubation period, initial pH values, and their variations during incubation period on FPase production by correlation analysis. Fpase production by *A. neoellipticus* was in strong postive correlation (0.9) with initial pH values. Never the less, weak postive correlations characterized the Fpase with incubation period (in days) while negative correlations characterized Fpase with variations taking place individually under each initial pH value getting stronger at pH 7 to be -0.7 except pH 4.

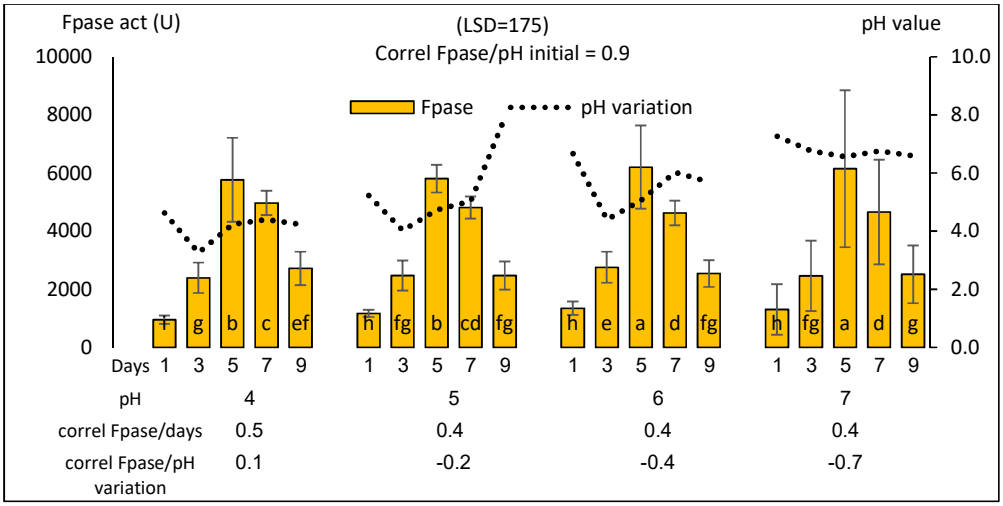

Fig. 1: Effect of initial pH value on cellulytic activity during 9 day.

3.3.2. Optimum N source

The optimization of N source included studying the impact of two inorganic and two organic types on cellulase production, as illustrated in Figure (2). Cellulase production was conducted at optimum initial pH 6, as previously noted in section [3.3.1.]. *A. neoellipticus* achieved its maximum cellulytic activities statistically using organic nitrogen sources than inorganic ones recording 8495U (LSD=198) with yeast ext. Correlations between Fpase activity and incubation period fluctuated under each N source but characterized by strong negative correlation of -0.7 in case of under NH_4NO_3 as N source.

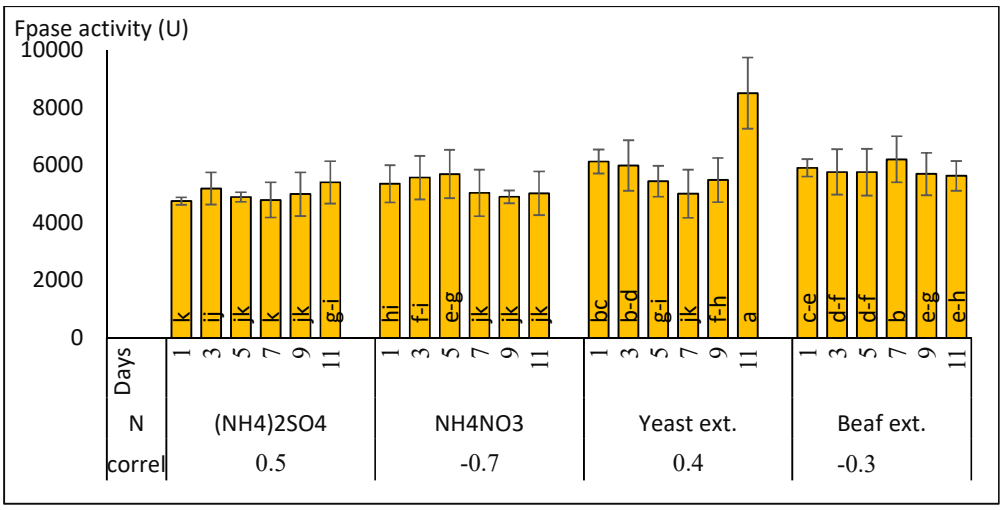

Fig. 2: Effect of initial N source on cellulytic activity during 11 day

3.3.3. Optimum C:N ratio

The growth media was supplemented with yeast extract in different C:N ratios, as illustrated in Figure (3). *A. neoellipticus* achieved its best cellulytic activity when C:N ratio was 1:0.9 to be 8238 U after 7 days confirmed by statistical analysis, where LSD=142. Progress in growth period had positive effect on Fpase activity according to positive correlations recorded among all C:N ratios.

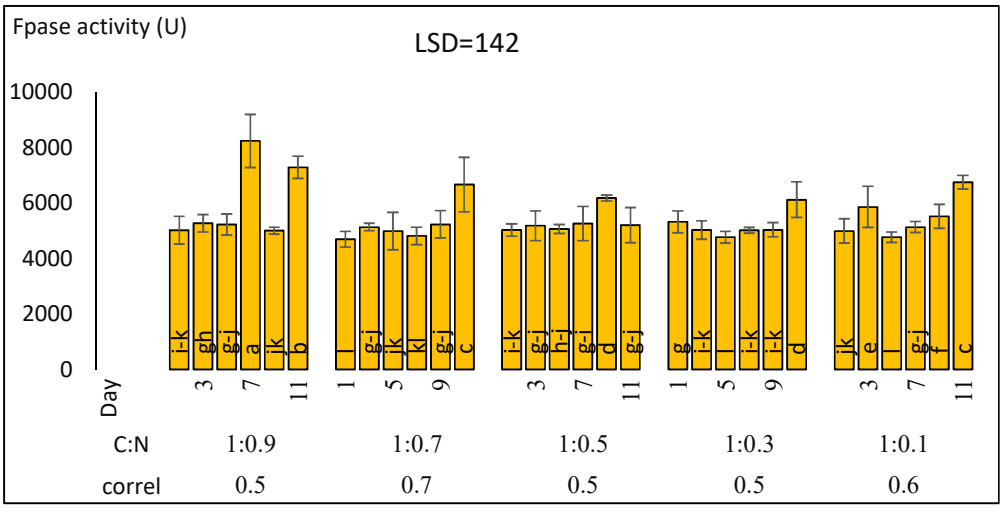

Fig. 3: Optimizing C: N ratio for maximum cellulase production

3.4. Saccharification

Cellulase production was conducted under initial pH=6 using beef extract as N source and C:N ratio adjusted at 1:0.9 targeting maximum yield regardless to period needed, after which the resulting raw cellulase was lyophilized to be used in saccharification of delignified RS in test tubes and flasks.

3.4.1. Optimum Saccharification conditions in test tubes

The lyophilized raw cellulase produced by *A. neoellipticus* showed three trends in response to incubation period, pH and temperature when applied in saccharification of treated RS, as shown in Table (2). In all cases, the saccharification was increasing as incubation period progressed. RS saccharification was statistically better under pH 6 and 7 at both temperatures 40°C and 50°C than with pH 5, where numerically best saccharification was under pH = 7 at 50ºC. The correlations shown in Table (3) showed the effect of both pH and temperature on saccharification % of treated RS by

cellulases from *A. neoellipticus*, after 4 days of saccharification. Saccharification correlations with increasing pH under both temperatures 40ºC and 50ºC were strongly positive to be +0.8 and +1.0, respectively, as shown under case I. Regarding the influence of temperature at each pH, the correlations were strongly negative at both pH values 5 and 6 to be -1.0, while it tended to be strongly positive at pH 7 to be +1.0.

Table 2: Effect of temperature and pH on RS Saccharification

T°C	pH	day	Sacch %	±	SE	Rank
40	5	1	7.47	±	0.04	d
		4	11.41	±	0.48	b
	6	1	7.70	±	0.35	d
		4	15.43	±	1.07	a
	7	1	7.58	±	0.22	d
		4	14.68	±	0.87	a
50	5	1	7.41	±	0.04	d
		4	10.40	±	0.04	bc
	6	1	10.07	±	0.78	bc
		4	15.02	±	0.94	a
	7	1	8.36	±	0.04	cd
		4	16.44	±	1.35	a

LSD = 1.994

Table 3: Correlation of Sacch % against each pH value and temperature degree

T°C	pH	Correl I	pH	T°C	Correl II
40	5	+0.8	5	40	-1.0
	6			50	
	7		6	40	-1.0
50	5	+1.0		50	
	6		7	40	+1.0
	7			50	

Correl I: Correlation between sacch% and initial pH under each incubation temperature
Correl II: Correlation between sacch% and incubation temperature under each initial pH

3.4.2. Optimum Saccharification conditions in Flasks

Saccharification of treated RS by the lyophilized cellulases was subjected to study the effect of scaling up from test tubes to flasks. The results shown in Table (4) demonstrated the saccharification % variation in accordance to incubation time at conditions that previously were proved to be optimum from results obtained in section 2.1., including incubation for 13 days at 40°C (pH=6) and 50°C (pH=7), respectively. The saccharification % of RS at 40°C / pH=6 fluctuated during incubation period achieving its highest result after 4 days to be 72.19 %, according to statistical analysis where LSD = 6.749, increasing 4.7 times than that recorded in test tube test. The fluctuation was proved by the week correlation of saccharification % in accordance to days of incubation period to be -0.2. On the other hand, saccharification at 50°C / pH=7 recorded 62.08% after 4 days increasing than that in test tube by 3.8 times, while recording its best after 13 days to be 89.11 based on statistical analysis where LSD= 10.973, increasing by 5.4 times that in the test tube test. Added to that, there were no fluctuations as correlation of saccharification % in accordance to days of incubation period was strong positive to be +1.0. The best of all saccharification result statistically where LSD = 8.8 was at 50°C / pH=7 after 13 days.

Table 4: Optimizing saccharification of RS (1g/70ml or 1.43%) in flasks by lyophilized enzyme (25U/flask) from *A. neoellipticus*

Saccharification conditions	days	Sacch%	±	SE	Rank [1]	Rank [2]	Rank [3]	Correl
40°C/ pH=6	2	68.08	±	1.74	c-f	ab		-0.2
	3	69.91	±	1.94	cd	ab		
	4	72.19	±	5.90	bc	a		
	5	63.44	±	8.30	d-g	bc		
	6	59.50	±	2.52	fg	c		
	7	58.40	±	1.01	g	c		
	10	63.50	±	4.98	c-g	bc		
	13	68.38	±	7.70	c-e	ab		
50°C/ pH=7	2	60.08	±	6.20	e-g		c	+1.0
	3	62.46	±	6.00	d-g		c	
	4	62.08	±	5.70	d-g		c	
	5	63.34	±	7.40	d-g		c	
	6	65.61	±	1.53	c-g		c	
	7	68.82	±	1.48	c-e		bc	
	10	79.68	±	2.00	b		ab	
	13	89.11	±	10.06	a		a	

1- LSD_1 for 40 and 50°C = 8.753
2- LSD_2 for 40°C= 6.749
3- LSD_3 for 50°C= 10.973
Correl: Correlations between saccharification % and incubation period in days.

Fermentation in bottles

3.4.3. Optimization

Depending on previous saccharification results in section 2.3., the RS saccharified at 50°C /pH=7 by the lyophilized cellulases was used in fermentation bottle as mentioned in materials and methods so that each bottle contained approximately 10.23% reducing sugar where it was mixed with the sterile fermentation medium. Fermentation was carried out for 48 hours, where the whole period was subclassed alphabetically to 4 sectors a, b, c and d representing development of fermentation period, as shown in Table (5). The statistical analysis concerning ranking the whole test results was labelled R^{all} (LSD= 6.628) while those ranks calculated individually for pH 4, 5 and 6 were labelled R^4, R^5 and R^6, having LSD of 4.642, 2.620 and 10.505, respectively.

According to those results, the fermentation efficiency % increased parallel to fermentation period under each pH value, where it didn't exceed 13% up till 45 hr under both pH values 4 and 5, achieving their maximum after 48 hr to be 79.4 and 98.1 %, respectively. Fermentation efficiency % at pH=6 exceeded 50% after 30 hr and reached after 48 hr to be 99.2% where its ethanol yield was close to the ideal theoretical yield.

Table 5: Fermentation efficiency in bottles as affected by initial pH

pH		hr	fer eff %	±	SE	R^{all}	R^4	R^5	R^6	C1	C2
		1	11.8	±	0.10	ij	b				
		3	10.3	±	0.09	j-m	b				
	a	6	9.8	±	0.05	j-m	b-d				
		9	5.2	±	0.15	k-m	de				
		12	4.9	±	0.07	lm	e				
		15	5.0	±	0.06	lm	e				
		18	4.7	±	0.02	m	e				
	b	21	4.8	±	0.02	m	e				
4		24	5.6	±	0.11	k-m	c-e			0.4	
		27	4.9	±	0.01	lm	e				
		30	4.8	±	0.07	lm	e				
	c	33	4.7	±	0.04	m	e				
		36	10.8	±	0.45	i-l	b				
		39	10.2	±	0.17	j-m	bc				
	d	45	10.2	±	0.10	j-m	b				
		48	79.4	±	6.43	b	a				
		1	9.9	±	0.07	j-m		c			
		3	10.6	±	0.27	j-m		bc			
	a	6	10.4	±	0.08	j-m		bc			
		9	5.7	±	0.05	k-m		d			
		12	5.7	±	0.10	k-m		d			
		15	6.0	±	0.24	j-m		d			
		18	5.5	±	0.05	k-m		d			
	b	21	5.8	±	0.06	k-m		d			
5		24	6.1	±	0.09	j-m		d		0.5	0.9
		27	6.2	±	0.04	j-m		d			
		30	6.7	±	0.56	j-m		d			
	c	33	6.3	±	0.29	j-m		d			
		36	11.5	±	0.17	i-k		bc			
		39	11.7	±	0.12	i-k		bc			
	d	45	12.6	±	0.21	hi		b			
		48	98.1	±	3.55	a		a			
		1	29.3	±	0.07	g			gh		
		3	24.0	±	5.18	gh			hi		
	a	6	20.7	±	1.18	h			hi		
		9	18.1	±	0.68	hi			i		
		12	21.0	±	0.43	h			hi		
		15	23.6	±	0.30	gh			hi		
		18	28.6	±	1.34	g			gh		
	b	21	36.1	±	1.64	f			fg		
6		24	37.4	±	1.99	f			e-g	0.9	
		27	45.4	±	2.88	e			d-f		
		30	55.0	±	2.82	d			d		
	c	33	47.4	±	1.69	e			de		
		36	71.8	±	1.95	c			c		
		39	85.3	±	9.75	b			b		
	d	45	80.1	±	6.84	b			bc		
		48	99.2	±	3.34	a			a		

Ranks for pH values 4, 5, 6 and all were R^4 R^5 R^6 and R^{all} having LSDs 4.642, 2.620, 10.505 and 6.628, respectively. Correlations included C^1 between fer eff % and period under each initial pH, while C^2: between the three initial pH values and their maximum fer eff % achieved.

Correlations were held for Ethanol fermentation efficiency against initial pH values and period in hours. Depending on those results in section 4.1.1., it could be advised to begin harvesting the ethanol produced after 30 hr and further at pH=6 at period sectors c and d, as production continued efficiently more than that at pH 4 or 5, as the correlations between fermentation efficiency and period

calculated under each pH value (C1) presented in Figure (4) proved to be strongly positive at pH 6 to be 0.9 more than that at pH 4 and 5 to be 0.4 and 0.5, respectively. Another correlation calculated between maximum fermentation efficiency % achieved under each pH value and those pH values to be strong positive 0.9 proving that the higher the pH value the higher the fermentation efficiency could be achieved.

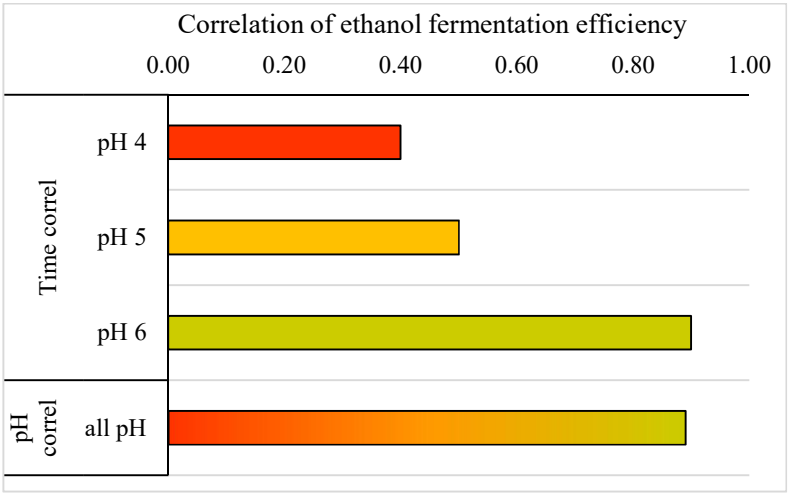

Fig. 4: Correlations of ethanol fermentation efficiency with either fermentation period in bottles under each initial pH value and with initial pH values

3.4.4. Rate of ethanol production

The ethanol production rate revealed both the production magnitude and the loss of ethanol in accordance to fermentation period as shown in Table (6). The production rate after 1 hr recorded 1.212, 1.013 and 3.001 g ethanol/bottle/hr. As fermentation progressed, fluctuations in production rates resulted in occasional losses in produced ethanol appearing as negative production rates that achieved 2.358, 2.917 and 0.652 g ethanol/bottle/hr at the end of fermentation period, under pH values of 4, 5 and 6, respectively. Obvious increase in production rates under pH 4 and 5 nearly by 200% and 290%, respectively, when comparing first hour with final 48 hr fermentation rates. Under pH 6 the first hour fermentation rate decreased by more than 78%, regardless to the accumulation in final results.

Table 6: Rates of ethanol production during fermentation in bottles

hr	Rate of ethanol production (g/bottles/hr.)		
	pH 4	pH 5	pH 6
1	1.212	1.013	3.001
3	-0.078	0.035	-0.271
6	-0.018	-0.008	-0.113
9	-0.155	-0.160	-0.090
12	-0.012	0.000	0.099
15	0.004	0.011	0.088
18	-0.009	-0.015	0.172
21	0.001	0.010	0.255
24	0.028	0.010	0.043
27	-0.022	0.003	0.275
30	-0.004	0.016	0.328
33	-0.003	-0.015	-0.259
36	0.207	0.177	0.831
39	-0.023	0.009	0.461
45	0.003	0.028	-0.179
48	2.358	2.917	0.652

3.5. Fermentation in fermentor

The 2L fermentor was first adjusted to carry out saccharification of 30g treated RS using 1.5L buffered lyophilized cellulases (0.5M/pH=7) with enzyme load of 25U/g RS and incubated at 50°C with agitation of 100 rpm. The process had been carried out for a period to give when mixed after wards with the sterile fermentation medium a final glucose concentration of 10.3%. The initial pH was adjusted at 6 while incubation at 35°C was carried out with no agitation and no introduced aeration. Samples were taken every 1 hr for 8 hr in the first day and also in the second day and lastly after 48 hr as shown in Table (7). The fermentation efficiency in the first day achieved 64% and then decreased down till 4 hr after which it continued increasing up to 84% at 8 hr. In the second day an obvious decrease down to 63% followed by fluctuating increase achieving its maximum at 29 hr with a final fermentation efficiency of 100% after 48 hr.

3.5.1. Rate of ethanol production

The rate of ethanol production shown in Table (7) clarified the cause of fluctuation in fermentation efficiency as the production rate parallelly fluctuated too. Nevertheless, the production rate had an apparent loss in ethanol specifically after 2, 4, 24, 27, 30 and 48 hrs where negative results were recorded. Never the less, the fluctuation prolonged the accumulation period needed to achieve the 100% fermentation efficiency after 48 hr.

Table 7: Saccharified RS fermentation in 2L fermentor

Days	Hours	fermentation efficiency %	Correl[1]	Correl[2]	rate of ethanol production (g/fermentor/hr)
1st	1	63.6	0.8	0.4	65.0
	2	36.4			-27.8
	3	47.3			11.2
	4	32.3			-15.3
	5	40.2			8.0
	7	44.3			4.2
	8	84.1			40.7
2nd	24	62.8		0.4	-1.4
	25	89.2			26.9
	26	89.8			0.7
	27	65.5			-24.9
	28	69.8			4.4
	29	93.1			23.8
	30	89.3			-3.9
	31	85.2			-4.2
Last	48	100.0			1.0

Correl[1]: correlation between fer eff % and the whole fermentation period
Correl[2]: correlation between fer eff % and specified fermentation period (day)

3.6. Compared fermentation efficiencies in bottle and in fermentor

The comparison of fermentation efficiencies of reducing sugar by *S. cerevisiae* in both the 200ml fermentation bottles and 2L fermentor was crucial to emphasize the effect of fermentation volume and accordingly the effect of *S. cerevisiae* inoculum size on this process. As illustrated in Figure (5), the fermentation efficiency in fermentor was higher than that in fermentation bottle under the same fermentation conditions. Fermentation finally produced 91g ethanol (115ml) from 1.8L medium containing 180g glucose from saccharified RS in the 2L fermentor.

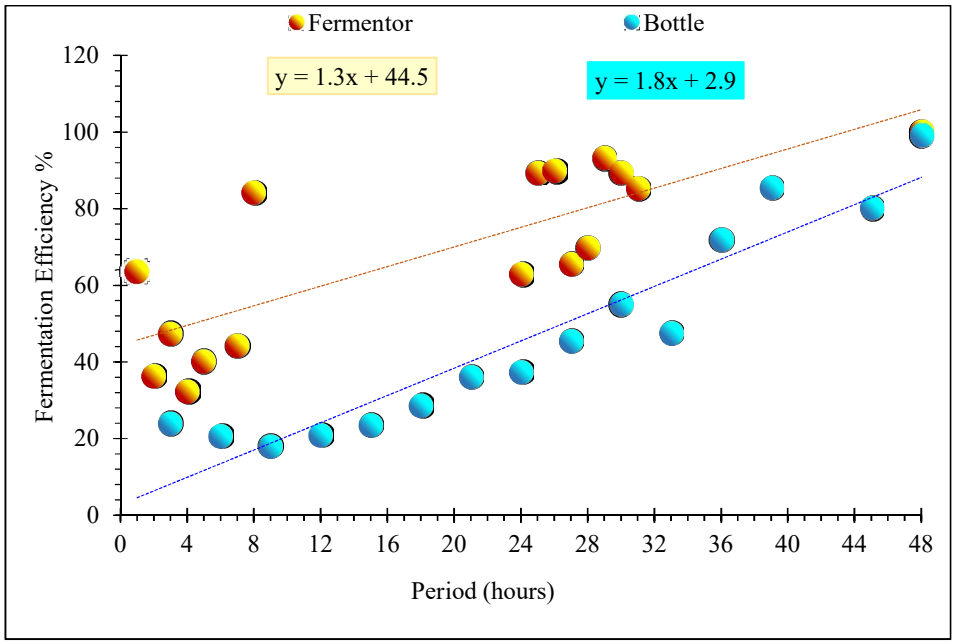

Fig. 5: Comparing fermentation efficiencies in bottle and fermentor at pH 6

3.7. GC-MS analysis

Fermentation final sample was assessed qualitatively by GC-MS analysis, as the constituents in the mass spectrum fragmentation pattern obtained by electron ionization (EI) were compared with those stored in Wiley and NIST Mass Spectral Library data, as shown in Table (8) and illustrated in Figure (6). Cation fragments appearing at specific m/z segmented from the main parent molecule by losing specific part as shown in Table (8).

Table 8: Peak list for the sample spectrum structure

m/z	Abundance	Relative int.	Cation fragment		lost
30.10	115791	5.7			
31.06	2044325	100.0	Fragment ion	$(CH_2OH)^+$	CH_3
32.04	40105	2.0			
41.00	33334	1.6			
42.00	125874	6.2			
43.00	296680	14.5			
44.10	30388	1.5			
45.07	1311586	64.2	Fragment ion	$(CH_3CH_2O)^+$ $(CH_2CH_2OH)^+$	H
46.09	497204	24.3	Parent molecular ion	$(CH_3CH_2OH)^+$	e

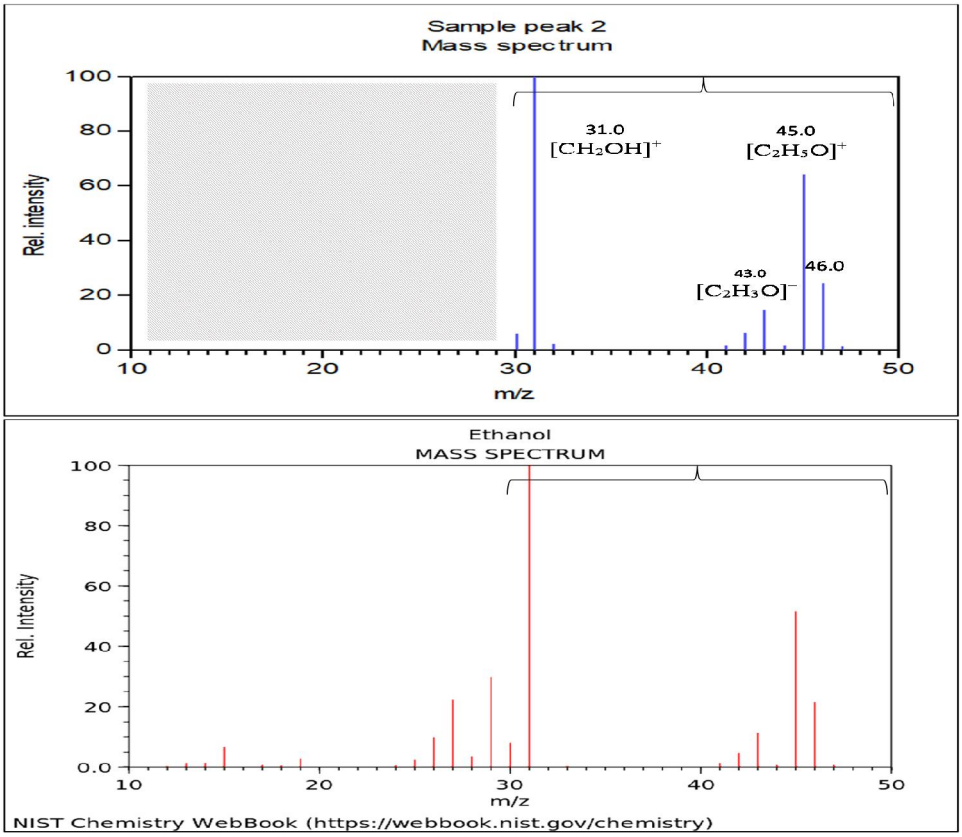

Fig. 6: GC-MS Spectrum of sample compared to that of ethanol reference from in Wiley and NIST Mass Spectral Library data.

4. Discussion

4.1. Isolation and genetic identification

The isolated fungal candidate from compost pile was identified as *A. neoellipticus* following members of *Aspergillus species*: section Fumigati illustrated in the phylogenetic tree. Its ecological existence was reviewed in many studies stated by Sugui *et al.,* (2015) including soil, organic debris and compost in high abundance, characterized by its ability to survive wide range of temperature between 12ºC to 65ºC while growing optimally at 37ºC.

4.2. Rice straw delignification

Rice straw delignification pretreatment in the present study was essential for removal of various physical and chemical barriers, swelling of fibers to allow more microbial cellulolytic enzymes to penetrate into the its cell wall structures resulting in efficient enzymatic hydrolysis (Weimer and Weston, 1985; Saddler *et al.,* 1993). Similar to the presented results, Kaur *et al.,* (2017) applied pretreatment to rice straw resulting in less lignin and more holocellulose.

4.3. Cellulase production

The agitation applied at 150 rpm during cellulase production in the present study was crucial for cellulase yield, enabling good levels of dissolved oxygen needed by the fungus without damaging its mycelial structure, based on findings of Sarkar and Aikat (2014). They stated before that 150rpm was optimum for cellulase production by *A. fumigatus*, above or below which the enzyme production decreased. Added to that, the amounts of cellulases released into the medium during growth of *A. fumigatus* were found to be dependent on many parameters including pH, nitrogen source, C:N ratio and temperature (Lee and Fan, 1982).

A. neoellipticus in the present study achieved its maximum cellulase production at pH 6, which lied in the range of optimum pH values from 3 to 7 that had been reported in many studies on

cellulase production by several fungi including *A. niger, A. fumigatus and Trichoderma longibrachiatum* (Stewart and Parry, 1981; Folakemi *et al.,* 2008; Nibedita and Kaustav, 2014). The pH of the medium was found to have an effect on the mycelial growth and enzyme production due to its role in transport of various components across the cell membrane as revealed by Sarkar and Aikat (2014).

Also, cellulase production was dependent on the nature of nitrogen source in the culture medium. Yeast extract 6% resulted in significant Fpase in the present study, as it was reported to promote microbial growth due to its amino acids content (Li *et al.,* 2014). In previous studies, various inorganic and organic nitrogen sources were tested for their effect on cellulase production where it was found that the maximum enzyme activities were achieved with the organic nitrogen sources peptone, tryptone, beef extract and yeast extract (Daroit *et al.,* 2007; Nibedita and Kaustav, 2014; Sarkar and Aikat, 2014). However, some other studies have reported inorganic nitrogen sources, specially (NH4)2SO4 of concentration 1%, resulting in higher cellulase production by *A. terreus* (Stewart and Parry, 1981; Kalogeris *et al.,* 2003; Ismail and Hassan, 2020).

In the present study, the increase in released glucose might be due to cellulose availability in the medium, while observed decrease in glucose production might be explained as a result of an inhibitory effect of accumulated cellobiose and cellodextrins of lower polymerization degree that bound specifically to the enzymes produced in the medium (Langsford *et al.,* 1984; Folakemi *et al.,* 2008). Added to that, the decrease in glucose production might be due to clumping of cells that led to many consequences including reduction in the uptake of sugar and oxygen, decrease in released enzyme and sugar depletion from its substrate used in the medium (Srivastava *et al.,* 1987; Brien and Craig, 1996).

On the other hand, optimum period required for *A. neoellipticus* all over the optimization studies extended from 3 to 7 days. Sarkar and Aikat (2014) and Singla *et al.,* (2018) found that maximum cellulase production by *A. fumigatus* applied on different agricultural residues was observed after 3 to 5 days.

4.4. Saccharification

Similar to the present study where *A. neoellipticus* was used in submerged culture to utilize rice straw for cellulase production, Sarkar and Aikat (2014) used *A. fumigatus* as well. In both studies, the crude extract as source of cellulase was used to hydrolyze pretreated rice straw.

Observed decline in hydrolysis rates held in flasks in accordance to time might be due to depletion of more amorphous substrates as the residue resulting from hydrolysis of cellulose became more crystalline and resistant (Ghose, 1987; Converse, 1993). Besides, as product inhibition discussed before in cellulase production, the accumulated cellobiose and glucose resulting from the cellulose hydrolysis might had exerted competitive inhibitory effect on the cellulolytic enzymes and/or enzyme inactivation (Lee and Fan*,* 1982; Ghose, 1987).

Any change in pH of medium observed in the present study led to changes in the ionic groups at their active sites and their three-dimensional shape that consequently being more or less active in accordance to certain pH range, so ever it was the introduced as initial pH or changes in pH happening during incubation period (Manonmani and Sreekantiah, 1987; Shuler and Kargi, 1992). The increase in incubation temperature to 50ºC was suggested that it pushed the reactant molecules to gain kinetic energy that led to more productive collision in accordance to time, which means better production at higher temperatures, as stated by Segel (1976).

Finally, increasing pH value and temperature had positive correlation of +1.0 to achieve the highest saccharification %, which increased in flasks compared to that in the test tube under best conditions recorded. Never the less, in spite that the enzyme load introduced was decreased in flask than test tubes by 1/10-fold, the increase in volume from 5ml up to 70 ml led to dilution of reactants and products that might had eliminated the inhibitory effect of accumulated cellobiose and glucose resulting from the cellulose hydrolysis that were the main causes for competitive inhibition as discussed before.

4.5. Fermentation by yeast (SSF)

In previous study, Laopaiboon *et al.,* (2007) found that the optimum sugar concentration in batch fermentation by *S. cerevisiae* was 190 g/L. Taking in consideration that ethanol production

(yield) in batch fermentation could be elevated by the increase in initial sugar concentration used, subsequently longer fermentation period would be needed which led production cost to increase (Zabed *et al.,* 2014). Based on those findings, using sugar concentration of 100g/L in the present study would be considered half the way to optimum production. On the other hand, Louhichi *et al.,* (2013) had found that the optimal ratio of sugar to microorganism was 6.7 (200:30 g/L) which comprised nearly half that in the present study, as the ratio of sugar to microorganism used was 12.5 (100:8 g/L). Temperature was maintained at 35˚C to be optimum as recommended by Ballesteros *et al.,* (2004) and Phisalaphong *et al.,* (2006).

The optimum pH for maximum fermentation efficiency % in the present study was recorded at pH 5 and 6**.** Similarly, Abouzied and Reddy (1986) had reported that the ethanol production by *S. Cerevisiae* was optimal at pH 5 to pH 6. On the same trend, Togarepi *et al.,* (2012) reported maximum rate of ethanol production achieved by *S. cerevisiae* was at pH 6.

Due to variations in pH during fermentation, H^+ concentrations in fermentation broth can change the total charge of plasma membrane affecting the permeability of some essential nutrients into the cells, thus it was found that pH range 4.0–5.0 was optimum for *S. cerevisiae* for ethanol production (Raphaelian, 1986). Similarly, when compared to fermentation in bottles held in the present study, the increase in fermentation rates under pH 4 and 5 were higher than pH 6 at the end, in spite that fermentation efficiency under pH 6 was better due to accumulation of ethanol produced.

In regard to fermentation time, the statistical correlation describing whole period was strongly positive (+0.8) proving the positive influence of time on fermentation efficiency. Added to that, fermentation period affected fermentation rate as well with observed occasional fluctuations. Zabed *et al.,* (2014) stated that shorter time in fermentation caused inadequate growth of microorganisms that subsequently caused inefficient fermentation, while longer fermentation time caused toxic effect on microbial growth especially in batch mode due to the accumulation of ethanol increasing its concentration in the fermented broth. Nadir *et al.,* (2009) found that *S. cerevisiae* produced the highest ethanol concentration after 64 h but dropped after 72 h of fermentation. On the other hand, Rabeya *et al.,* (2020) found that fermentation process in bottles was time-dependent as the bioethanol production only increased for 24 h and decreased afterwards.

Compared to present study, the ethanol production rate was maximum in bottles after 36 h (0.831 g/bottle/hr ≈ 0.5%, w/v) and in fermentor after 8 h (40.7 g/fermentor/hr ≈ 2.3%, w/v), in which the production rate in fermentor recorded 4.6-fold that in bottles in less than 1/4 time. This made the ethanol harvesting during fermentation period more feasible from fermentor than bottles, if it was intended to be used.

4.6. GC-MS analysis

As explained by Raphaelian (1986), the prominent peak at m/z 45 corresponded to the loss of a hydrogen radical (H•) from the ionized ethanol molecule (parent molecular ion), while loss of H_2 formed the m/z 43 ion. On the other hand, breaking of the β bond gave resonance stabilized cation shown as prominent peak at m/z 31, which was relatively large in comparison to other peaks in the spectrum confirming the primary alcohol. As shown in Figure (6), the primary alcohol was confirmed by Wiley and NIST Mass Spectral Library data.

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