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Production of exo-inulinase from *Aspergillus niger* and *Candida oleophila* for degradation of chicory root inulin and ethanol production

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ABSTRACT

Exo-inulinase is an enzyme that is capable of breaking down inulin to produce fructose molecules. This enzyme produced by various fungal strains belonged to genera of *Aspergillus, Trichoderma, Penicillium, Saccharomyces* and *Candida.* Two fungal strains (*Aspergillus niger* and *Candida oleophila*) were selected among of 12 the tested strains which gave the highest inulinase activity (I) on chicory root, giving 40 and 35 mm of clear zone diameter on an agar plate and 55.93 and 46.38 U/ml in broth medium, respectively. The selected two also strains gave the highest invertase activity (S) on sucrose 95.04 and 86.94 U/ml with high values of I/S ratio (0.59 and 0.53). Both enzymes were better produced using co-cultures of *A. niger* and *C. oleophila* than using single culture. Optimization of fermentation parameters (11 factors) for both enzymes production from the tested co-cultures was carried out using the design of Taguchi orthogonal array (OA). Results showed that 5 (inulin, corn steep liquor (CSL), temperature degree, pH and KH_2PO_4) and 3 (inulin, CSL and temperature degree) were most significant factors affecting on production of inulinase (102.3 U/ml) and invertase (139.5 U/ml)which were closely related with predicted values of 103.6 and 140.1 U/ml, respectively. Fungal inulinase was applied to chicory inulin hydrolysis compared to pure inulin for the production of fructose with content of 61% and 75%, respectively, after 60 min of reaction time. It was also found that ethanol production by inulinase was achieved using 3 processes, which were direct conversion (DC) of inulin into ethanol by mixed cultures *A. niger* and *C. oleophila,* separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). The SHF and SSF methods were inoculated with *Saccharomyces cerevisiae* for fermentation sugars into ethanol. The highest ethanol yield was recorded during SSF process (15.60 g/L) compared with SHF (7.36 g/L) and DC (2.83 g/L) processes.

***Keywords*:** Inulinase, Invertase, *Aspergillus niger*, *Candida oleophila, Saccharomyces cerevisiae*, ethanol production, inulin hydrolysis

Introduction

Inulin is polysaccharide that classified to a very important section of carbohydrates recognize as fructans found in many plants such as asparagus, chicory, agave, dahlia, coffee, dandelion, garlic, Jerusalem artichoke, globe artichoke, onion and jicama which they store these polymers as a carbon and energy sources. It consists of many units of fructose linked to each other through β-2, 1-glycosidic bonds (Kango and Jain, 2011; Hughes *et al.,* 2017)

Inulinases are specific enzymes which catalyze the hydrolysis of the plant inulin to fructose and inulo-oligosaccharides (Bonciu *et al.,* 2011). There are 3 various kinds of β-fructofuranosidases hydrolyzing inulin including invertase, exo- and endo- inulinases (Rossi *et al.,* 2005). Each of exo-inulinase (2,1-D-fructan fructanohydrolase, E.C. 3.2.1.7) and invertases (β-D-fructofuranoside-fructohydrolase, E.C. 3.2.1.26) are β-fructosidases which hydrolyse the β-D-2, 6- fructan linkages release fructose from the fructosyl terminal, while endo-inulinases act on the internal glycosidic linkages(Coitinho *et al.,* 2010).

Many of filamentous fungi and yeast produce commercial inulinases using submerged culture including *Aspergillus* spp., *A. tubingensis*, *Penicillium rugulosum, Rhizoctonia* spp.,*Mucor circinelloides, Pichia guilliermondii, Saccharomyces cerevisiae, Candida guilliermondii* and

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Kluyveromyces marxianus (Chi *et al.,* 2009; Bonciu *et al.,* 2011; Jain *et al.,* 2012; Trivedi *et al.,* 2012; Meena *et al.,* 2018; Prangviset *et al.,* 2018; Singh *et al.,* 2018).

Agro-industrial tannins wastes (chicory root and Jerusalem artichokes) have generally environmentally negative effect which consider a pollution source. So, bio-conversion of these residues into valuable important products useable in industrial applications such as production of enzymes and fermentable sugars (fructose syrupy) in addition to bioethanol (Bonciu *et al.,* 2011; Chi *et al.,* 2011; Hughes *et al.,* 2017; Qiu *et al.,* 2018) can help minimize their negative effect on the environment.

Bioethanol is a renewable alternative to fossil fuels where it simplifies energy safety and decreases the emissions of greenhouse gas. Bioethanol is commercially produced by simple sugars fermentation from food inputs (corn or sugar cane, etc...) or agro-industrial wastes, which are called first or second generations bioethanol, respectively (Zabaniotou *et al.,* 2008; Ishola *et al.,* 2014). The first-generation bioethanol is a drawback due to the fact of its an excessive rate the place the raw materials got from the meals market and resulting in accelerated product value(Zabaniotou *et al.,* 2008; Ullah *et al.,* 2015). Whereas, the second generations bioethanol is more preferred because of the low cost of wastes and overcome environmental pollution, and considered as eco-friendly (Ishola *et al.,* 2014; Maitan-Alfenas *et al.,* 2015). Bioethanol can be produced from inulin by two methods namely, sequential hydrolysis and fermentation (SHF) and simultaneous saccharification-fermentation (SSF). SHF process is performed by two steps, firstly enzymes are applied to degrade the wastes, such as raw residues into simple sugars then produced sugars are fermented into ethanol (Rastogi and Shrivastava, 2017). This process has a disadvantage because both steps required optimal conditions and the hydrolytic enzymes employed can suffer from feed-back inhibition forms negative impact on this process productivity (Dussán *et al.,* 2016). On the other hand, SSF process is carried out by one step which both stages of enzyme hydrolysis and fermentation are achieved at the same time. This process has some advantages including reducing the cost and time of operation, where the simple sugars produced by yeast are simultaneously fermented to ethanol, and giving a high activity of the enzyme(Chandel *et al.,* 2012; Rastogi and Shrivastava, 2017). Moreover, the SSF process can achieve 10 to 30% increase in ethanol production over SHF process(Öhgren *et al.,* 2007). The SHF and SSF processes were carried out using *Saccharomyces cerevisiae* for ethanol production from cassava pulp and inulin rich raw materials, etc… without any pretreatment (Neagu and Bahrim, 2012; Zhu *et al.,* 2012).

Therefore, the purpose of the present investigation was to obtain an efficient inulinase-producing strain, and evaluate the enzyme activity for chicory roots inulin hydrolysis and fructose production as well as its application for ethanol production with various fermentation approaches.

Materials and methods

Microorganisms used

Nine fungal strains *Aspergillus terrus, A.niger, A. tubingensis* USMI03, *A. awamori*, *Trichoderma harzianum*, *T. viride*, *T. reesei, Penicillium chrysogenum* and *P.digitatum*, and three yeast strains *Saccharomyces* sp., *S. cerevisiae and Candida oleophila* obtained from Agricultural Microbiology Department, Faculty of Agriculture, Ain Shams University and were used for inulinase production in this investigation. Meanwhile*, S. cerevisiae* was also used for ethanol production. Thesestrains were maintained on potato-dextrose agar medium slants (Evancho *et al.,* 2001) at 27°C, and stored at 4°C.

Screening for the most efficient inulinase producing strains using diffusion method

This technique was described by Silvera *et al* (2018) which used for qualitative determination of inulinase activity by the tested strains. The tested strains were inoculated on inulin agar medium plate (Silvera *et al.*, 2018) in a Petri dish composited of (g/L): 10, inulin, 1.5, $NaNO_3$; 2, $(NH_4)_2SO_4$, 1, KH_2PO_4; 0.5, $MgSO_4.7H_2O$; 0.1, $FeSO_4.7H_2O$ and 20, agar. Plates were incubated at 27 °C for 72 h. After incubation, the plates were flooded with 5ml of 1% Lugol's iodine for 10 min to allow iodine to be absorbed. The clear zone formation around the microbial colonies was measured with a ruler which indicated the presence of inulinase activity hydrolysate inulin in the medium.

Submerged fermentation

Production of inulinase was performed in 250 ml plugged Erlenmeyer flasks containing 100 ml of inulin liquidinulin medium (was the same as inulin agar medium without adding agar) and inoculated with 4% standard inoculum of the fungal and yeast strains were 2.5x 10^7 spores/ ml and 1.8x10^8 colony forming unit (CFU) /ml, respectively. Inoculated flasks were incubated at 28°C on rotary shaker at 120 rpm for 72 h. At the end of fermentation, the samples (10 ml) were withdraw and centrifuged under cooling at 8496 xg for 10 min. The biomass was determined using pellets and each of inulinase, invertase and fructose yield were assayed in the cell-free supernatant as described later.

Effect of inoculums ratio on inulinase, invertase and fructose production by mixed cultures

This experiment was designed to assay the effect of inoculum size of mixed cultures of *A. niger* and *C. oleophila* on activities of inulinase and invertase and fructose production. The inoculum sizes of *A. niger* and *C. oleophila*(*v/v*) were 4, 6, 8 and 10% *(v/v)* with ratios of 0:4, 2:2, 2:4, 2:6, 2:8, 4:0, 4:2, 6:2 and 8:2% (*v/v*).

Statistical optimization of fermentation parameters for inulinase and invertase activity by co-cultures of *A. niger* and *C. oleophila*using Taguchi Orthogonal Array (TOA) Design

Taguchi orthogonal arrayL12 (2^11)experimental design, developed and analyzed by the statistical software package Design-Expert® software version 8.0. (2010), was used for selecting the most significant independent variables affecting inulinase and invertase production by mixed cultures *A. niger* and *C. oleophila*. An L12 orthogonal array in two levels (low and high) and 11 independent variables (inulin concentration, corn steep liquor (CSL) concentration, soybean meal (SBM) concentration, $(NH_4)_2SO_4$ concentration, $NaNO_3$ concentration, KH_2PO_4 concentration, pH, inoculum size, incubation period, temperature degree and agitation speed) was used consisting of 12 different experimental runs have been suggested by Roy (2001) as represented in Table 1.Experimental results were analyzed by analysis of variance (ANOVA) involving % main effect, *F*-value, *P*-value, determination coefficient (R^2), adjusted R^2, predicted R^2 and adequate precision and regression coefficients.

Table 1: Taguchi orthogonal array L12 (2^11) experimental design matrix, the results of actual values and analysis of variance (ANOVA) of factors affecting inulinase and invertase production from mixed cultures of *A. niger and C. oleophila*.

Run no.	A	B	C	D	E	F	G	H	J	K	L	Enzymes activity (U/ml)	
												Inulinase	Invertase
1	15	1.5	1.5	0	1.5	3	5.5	6	2	30	0	7.23	9.12
2	15	1.5	0	0.5	0	3	5.5	4	2	30	120	102.3	139.5
3	30	0	0	0.5	1.5	3	5.5	4	3	27	0	19.33	44.1
4	15	0	1.5	0.5	1.5	1.5	5.5	6	3	30	120	55.9	71.1
5	30	0	1.5	0	1.5	3	6.5	6	2	27	120	43.7	60.3
6	15	0	0	0	0	3	6.5	4	3	30	120	70.9	107.1
7	30	1.5	1.5	0.5	0	3	6.5	6	3	27	0	8.19	12.64
8	30	1.5	0	0	1.5	1.5	6.5	6	3	30	0	2.66	8.28
9	15	1.5	0	0.5	1.5	1.5	6.5	4	2	27	120	87.3	123.3
10	30	1.5	1.5	0	0	1.5	5.5	4	3	27	120	95.5	126.9
11	15	0	0	0	0	1.5	5.5	6	2	27	0	12.3	15.54
12	30	0	1.5	0.5	0	1.5	6.5	4	2	30	0	14.1	18.9
Analysis of variance (ANOVA)													
Inulinase yield	**F value**	179.66	50.29	109.74	9.09	0.01	20.06	25.61	0.12	17.13	39.35	11.61	
	p-value	0.01	0.02	0.06	0.09	0.94	0.05	0.04	0.76	0.07	0.02	0.08	
Invertase yield	**F value**	127.40	37.33	2.27	6.71	0.44	18.46	7.49	0.12	8.70	30.03	14.89	
	p-value	0.01	0.03	0.37	0.12	0.63	0.05	0.11	0.76	0.10	0.03	0.06	
R-Squared											0.99	0.99	
Adj R-Squared											0.97	0.96	
Pred R-Squared											0.80	0.72	
Adeq Precision											17.33	14.19	

A= Inulin concentration (g/L), B= corn steep liquor concentration (g/L), C= soybean meal concentration (g/L), D= $(NH_4)_2SO_4$ concentration (g/L), E= $NaNO_3$ concentration (g/L), F= KH_2PO_4 concentration (g/L), G= pH, H= inoculum size (%), J= incubation period (day), K= temperature degree (°C) and L= Agitation speed (rpm). Adj= adjusted, Pred. = predicted and Adeq= Adequate precision

Applications of *A. niger and C. oleophila* inulinase

Hydrolysis of inulin

The inulin hydrolysis was assayed according to Saber and El-Naggar (2009);1 ml crude inulinase was added to 0.25 ml (4% w/v) of inulin and chicory in 0.75 ml of sodium acetate buffer (pH 5.2) and incubated at 45°C for various time ranged from 0 to 120 min. Fructose content (%) was determined as a reducing sugar in the reaction mixture and inulin hydrolysis was calculated according equation as follows; Reducing sugar (%) / total sugar)x 100. Total sugar in hydrolyzed products was determined as described by Scott and Melvin (1953).

Ethanol production

Ethanol production in the current investigation was carried out through 3 processes in inulin modified medium by increasing inulin concentration up to 8%.

First process is direct conversion (DC) of inulin into ethanol by mixed cultures *A. niger* and *C. oleophila* (as control): the fermentation medium was inoculated with mixed cultures *A. niger* and *C. oleophila* for 48 h. which the mixed cultures secreted inulinase which degraded inulin to fructose then fermented to ethanol.

Second process is separate hydrolysis and fermentation (SHF): crude enzyme (supernatant) produced (separated) from 1st process was inoculated with the seed culture of the tested *S. cerevisiae* strain for 48 h at 120 rpm.

Third process is simultaneous saccharification and fermentation (SSF). Fermentation medium was inoculated with mixed cultures of *A. niger* and *C. oleophila* for inulinase production and *S. cerevisiae* for ethanol production in the same time for 48 h at 120 rpm

At the end of fermentation, the fermented cultures were centrifuged for 10 min at 8496 xg and 4 °C. The total and reducing sugars, ethanol concentration were determined in supernatant as described later. Ethanol productivity (g/L/d) and % Saccharification were calculated according to Lee (1996); Dysseler *et al* (1999), respectively, according to the following the equations:

Ethanol productivity (g/L/d) = Ethanol concentration (g/L) / Fermentation time (day).

% Saccharification = ((Reducing sugars (g/L) x 1.15)/ initial substrate concentration (g/L) x 100.

Analytical methods

Cell dry weight (g/L) was estimated by washing the pellets (biomass) about 3-times with distilled water then dried at 80°C until the weight was constated. Inulinase (I) and invertase (S) activities (U/ml) were assayed by the method described by Trivedi *et al* (2012). Briefly, this method was performed as follows; the mixture reaction consisted of 0.5 ml crud enzyme, 4.5 ml solution of 1 % (w/v) inulin or sucrose dissolved in sodium acetate buffer (0.2 M and pH 5). The mixture reaction was incubated at 60°C for 20 min. The reaction was stopped by incubated in the water bath at 100 for 10 min to inactivate the enzyme and then cooled at room temperature. The fructose liberated as end-product was assayed was determined by using 3,5 Dinitro salicylic acid solution (DNSA) method (Miller, 1959), using fructose as a standard curve. One unit (U) of inulinase or invertase was defined as the amount of enzyme required to produce 1 µmol of fructose/1 ml /1 min at 60°C.

The relationship between Inulinase/Invertase activities ratio was used to identify inulinase (Ohta *et al., 2*002) and calculated as follows

I/S Ratio= U/ml of inulinase activity/ U/ml of invertase activity.

Total sugars were analyzed by the same method after acid hydrolysis with 1 N HCl and heated at 100 min for 60min. The concentration of ethanol concentration (g/L) was estimated by using a redox back titration method (Iland, 2000) and was briefly mentioned as follows; the mixture reaction was added into a conical flask contained 20 ml of diluted fermented sample (supernatant) 1:25 with distilled water, 20 ml of potassium dichromate solution (0.04 N) and 10 ml of sulfuric acid concentrated (40%), then the reaction was mixed well and heated after covered with stopper at 45 C in water bath for 10 min and cooling the flask. After that, 2 g of potassium iodide was added to reaction and titrated with sodium thiosulfate solution (0.01 M) until the brown colour turned to pale yellow and then added 1 ml starch indicator solution (0.1%) which the reaction took blue-black colour for starch-iodine complex formation and continue titrating until blue colour disappears and reached to endpoint when the reaction turned to

clear green. The volume (end point) of sodium thiosulfate (the titer) was recorded to calculate the concentration of ethanol.

Statistical analysis

The data analysis was performed using software of IBM® SPSS® Statistics Server Version 23.0. (2015), which applied Duncan's test (Duncan's 1955) at 5% level to compare between mean values.

Results and Discussion

Results in Table 2 showed that extracellular inulinase was produced by different fungal and yeast strains classified into to genera of *Aspergillus, Trichoderma, Penicillium, Saccharomyces* and *Candida.* All the tested microbial strains (12 strains) were appeared on chicory root agar plates a clear zone around the growth of microbial colonies ranged from 10 to 40 mm of diameter zone. The clear zone formation indicated that tested strains capable to degrade inulin into fructose (Gong *et al*., 2008). Theses strains gave inulinase (I) and invertase (S) in a broth medium containing chicory root with activities ranged from 5.34 to 55.93 U/ml and 10.98 to 95.04 U/ml, respectively. The ratio of activity on inulin/sucrose (I/S Ratio) was ranged from 0.45 to 0.59 and the concentration of fructose was ranged between 1.02 and 11.42 g/L. This ratio showed the catalytic activity of these enzymes have been described by Kango (2008); Saber and El-Naggar (2009).

Table 2. Qualitative determination of inulin hydrolysis zone diameter on agar plate after stained with iodine solution and quantities determination of inulinase (I) and invertase (S) activities, I/S Ratio and fructose yield from different fungal and yeast strains in broth medium supplemented with 1% chicory inulin after 72h of incubation period using shake flasks.

Tested Fungi and yeast	Zone diameter (mm)	Inulinase yield (U/ml)	Invertase yield (U/ml)	I/S Ratio	Fructose yield (g/L)
Aspergillus terrus	25^{d}	20.92^{d}	39.98^{e}	0.52	3.75^{e}
Aspergillus niger	40^{a}	55.93^{a}	95.04^{a}	0.59	11.42^{a}
*Aspergillus tubingensis*USMI03	30^{c}	12.28^{f}	25.08^{f}	0.49	2.20^{f}
Aspergillus awamori	20^{e}	5.34^{g}	10.98^{i}	0.49	1.02^{g}
Trichoderma harzianum	20^{e}	34.20^{c}	74.70^{c}	0.46	6.54^{c}
Trichoderma viride	25^{d}	16.29^{e}	36.50^{e}	0.45	4.89^{d}
Trichoderma reesei	20^{e}	8.19^{g}	16.38^{h}	0.50	1.95f^{g}
Penicillium chrysogenum	25^{d}	13.00^{f}	27.68^{f}	0.47	2.94^{f}
Penicillium digitatum	10^{g}	34.11^{c}	68.04^{d}	0.50	6.08^{c}
Saccharomyces cerevisiae	25^{d}	9.91^{g}	22.14^{fg}	0.45	2.03^{f}
Saccharomyces sp.	15^{f}	14.37^{ef}	29.66^{f}	0.48	2.76^{f}
Candida oleophila	35^{b}	46.38^{b}	86.94^{b}	0.53	9.49^{b}

I= Inulinase and S= Invertase

- Values presented in the same column have the same letter, do not significantly differ from every different at 5 % level.

Out of 12 microbial strains, 2 most active strains of *A. niger* and *C. oleophila* were chosen which achieved a high significant ($p \leq 0.05$) activities of inulinase (55.93 and 46.38 U/ml) and sucrase (95.04 and 86.94 U/ml) with I/S ratio of 0.59 and 0.53 and content of fructose was 11.42 and 9.49 g/L, respectively. Whereas, the lowest enzymes activity and fructose yield were recorded by *A. awamori* and *T. reesei.* Rawat *et al.* (2015) found that 3 fungal strains of *A. niger* GNCC2655, *A. niger* ATCC 26011 and *A. awamori* MTCC 2879 gave maximum yield of inulinase followed by *Penicillium* sp. NFCCI 2768 and *Penicillium citrinum* MTCC 1256. Moreover, both strains of *A. niger* GNCC2655 and *A. awamori* MTCC 2879 recoded high activity of sucrase. Saber and El-Naggar (2009); Dinarvand *et al.* (2013) reported that maximum inulinase production by *A. tamari* AR-IN9 and *A. niger* ATCC 20611was attained after 72 and 96 h of incubation period, respectively.

From these results, it could be stated that the both strains of *A. niger* and *C. oleophila* as the most efficient strains for inulinase production were selected for further investigation.

Inulinase production from single and co-cultures of *A. niger* and *C. oleophila*

Biomass, fructose content, enzymes activity and relationship between inulinase and invertase activity (I/S Ratio) were registered in mono- and co-cultures of *A. niger and C. oleophila.* Results in Figure 1 clearly showed that there is a positive relationship between microbial growth and each of fructose yield and enzymes activity. It was observed that with the increase in cells growth, the yield of enzymes and fructose were increased. In single culture, *A. niger and C. oleophila* were inoculated with 4% of inoculum size. *A. niger* represented more increase about 20.3 and 20.6 & 9.3% in the production of fructose and enzymes (inulinase and invertase) than *S. cerevisiae,* respectively. Furthermore, Sirisansaneeyakul *et al.,* (2007)found that production of fructose from mold *A. niger* TISTR 3570 enzymes was significantly higher than from yeast *C. guilliermondii* TISTR 5844 enzymes. The effect of co-cultures of *A. niger* and *C. oleophila* with inoculum ratios of 2:2, 2:4, 2:6, 2:8, 4:2, 6:2 and 8:2% (*v/v*), respectively. Maximum cell growth (3.23 and 3.67 g/L), fructose yield (12.56 and 14.54 g/L) and enzymes activity (69.55 and 76.83 of inulinase and 98.52 and 102.02 U/ml of invertase) with I/S ratios been 0.71 and 0.75 were attained in fermentation medium inoculated with an inoculum size of 4 and 6% co-cultures (2: 2 % and 4: 2% of *A. niger*: *C. oleophila*). Fructose and enzymes production were decreased with increase the inoculum sizes to 8 and 10 %, it might be due to the depletion of nutrients and the cells produce curb (inhibitor) metabolites (Kashyap *et al.,* 2002; Shata *et al.,* 2014).

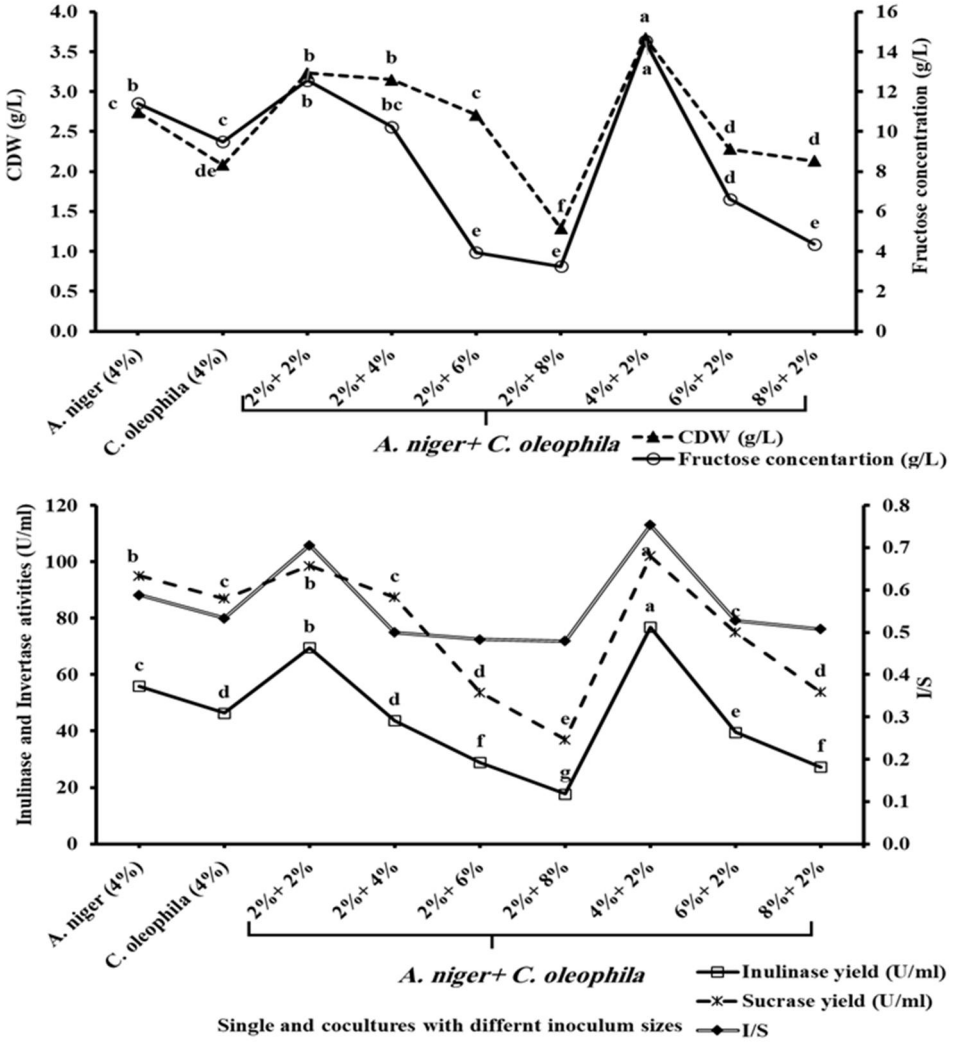

Fig. 1: Evaluation of cell dry weight, fructose yield, enzymes activity and I/S Ratio from monoculture (*A. niger and C. oleophila*) and co-cultures with various inoculation ratios using shake flasks at 27°C for 72h.

I= Inulinase and S=Invertase. Values presented in the same lines have the same letter, do not significantly differ from every different at 5 % level.

From these results, it could be observed that the mixed cultures produced a synergistic effect for inulinases production which an effect greater than the production of their single culture it might due to resulted from combined activity of invertase from both fungi, where the inulin hydrolysis demand to some activity of invertase to liberate the fructose molecules from the β (2,1)-linkages and resulting increase inulinase (Molefe, 2014).Similar results were observed also in literature(Hölker and Lenz, 2005; Ramesh and Reddy, 2013). Mixed cultures (4% of *A. niger* and 2% of *C. oleophila*) were chosen for further studies, which achieved an increase of fructose, inulinase and invertase yields about 27 or 53%, 37 or 66% and 7 or 17 % more than obtained from monoculture of *A. niger* or *C. oleophila*, respectively.

Statistical Optimization of inulinase and invertase production from co-cultures of *A. niger and C. oleophila* using Taguchi orthogonal array (TOA*)* design

The effect of 11 independent variables (inulin concentration, CSL concentration, SBM concentration, $(NH_4)_2SO_4$ concentration, $NaNO_3$ concentration, KH_2PO_4 concentration, pH, inoculum size, incubation period, temperature degree and agitation speed) on inulinases production by mixed cultures of *A. niger* and *C. oleophila* were tried using Taguchi (OA)design in 12 runs (Table 1). Data in Table 1 listed that the yields of inulinase were ranged from 2.66 to 102.3 U/ml of inulinase activity and ranged from 8.28 to 139.5 U/ml of invertase activity. The maximum yields of inulinase (102.3 U/ml) and invertase (139.5 U/ml) were recorded in experimental run 2 which variables setting were: inulin concentration of 15 g/L, CSL concentration of 1.5 g/L, SBM concentration of 0 g/L, $(NH_4)_2SO_4$ concentration of 0.5 g/L, $NaNO_3$ concentration of 0 g/L, KH_2PO_4 concentration of 3 g/L, pH of 5.5, inoculum size of 4%, incubation period of 2 days, temperature degree of 30°C and agitation speed of 120 rpm. Whereas, the lowest yields of inulinases were attained in experimental runs 1 and 8.

From an analysis of variance (ANOVA), indicated that the combination between variables at the same time was a significant impact on inulinase and invertase production which the model F-values of 39.21 and 27.90 implies and values of "Prob > F" less than 0.05 been 0.03 and 0.04, respectively (Data not shown). The ANOVA in Table 1 and Figure 2 demonstrated that 5 and 3 factors were significant impact with a very low probability value ($P \leq 0.05$)for inulinase (inulin concentration, CSL concentration, KH_2PO_4 concentration, pH and temperature degree) and invertase (inulin concentration, CSL concentration and temperature degree) production. Results also indicated that R^2 of the models was 0.99 which means that 99 % of the total variation is explained by the model. While the predicted R^2 was found to be 0.80 and 0.72 is in reasonable agreement for inulinase and invertase production with the adjusted R^2 been 0.97 and 0.96, respectively. Adequate precision was 17.34 and 14.19 of inulinase and invertase yields which measured the range of predicted values at design points to the average prediction error, respectively. A ratio greater than 4 is desirable.

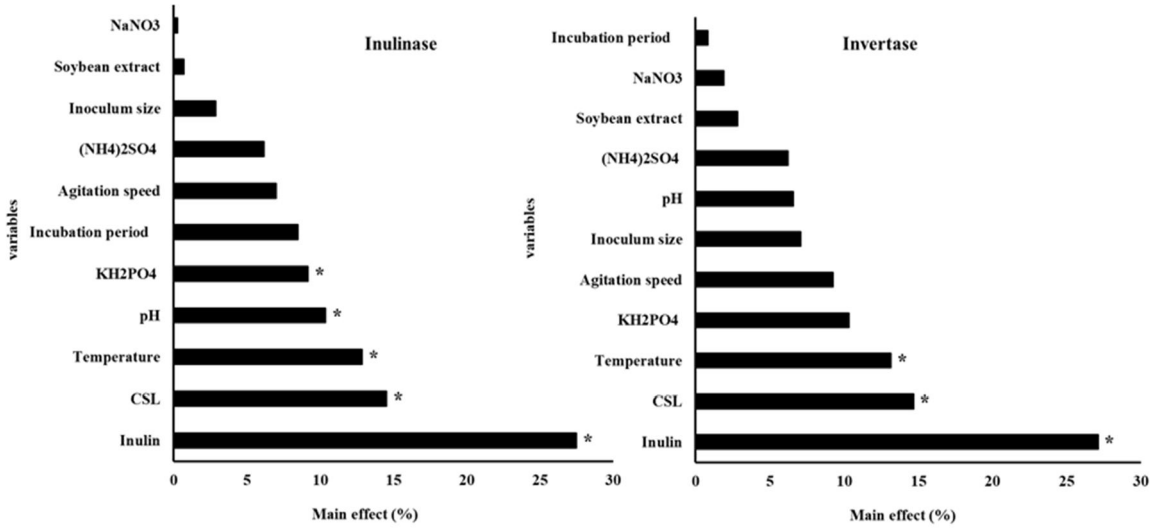

Fig. 2: Pareto graph showing effect of fermentation parameters on inulinase and invertase production from co-cultures of *A. niger* and *C. oleophila* based on the observation of Taguchi OA design.

The Pareto chart represented the order of significance of the variables affecting Inulinase and invertase production in TOA experimental design have been illustrated in Figure 2. Out of 11 variables, 5 variables of inulin concentration, CSL concentration, temperature degree, pH and KH_2PO_4 concentration) were the most significant factors (9.19-27.49 % main effect) on inulinase production. Whereas 3 variables (inulin concentration (27.12%), CSL concentration (14.68%) and temperature degree (13.17%) were the highest impact (13.17% - 27.12% main effect) invertase production. However, an anther factors affected inulinase and invertase production to a lower level (approximately 0.28- 8.48 and 0.84- 10.32 % main effect) than other 5 and 3 selected factors, respectively.

Regression coefficients analysis of the investigated variables exhibited that each of CSL, SBM, $NaNO_3$ and inoculum size was a positive impact on inulinase and invertase production except inoculum size was negative effect on invertase production. While each of inulin, $(NH_4)_2SO_4$, KH_2PO_4, pH, incubation period, temperature degree and agitation speed appeared a negative impact on the production of both enzymes. The predicted *vs.* actual values of the model is illustrated in Figure 3. There is a good correlation between the actual values and predicted values. Where the regression equations obtained after ANOVA were as follows:

Y Inulinase yield= 43.28- 25.68 (Inulin conc.)+13.59 (CSL conc.)+2.70 (BSM conc.)-5.78 ($(NH_4)_2SO_4$)+0.26 ($NaNO_3$)-8.551 (KH_2PO_4)-9.70 (pH)+0.66 (Inoculum size)-7.93 (Incubation period)-12.02 (Temperature degree)-6.53 (Agitation speed) Equation (3).

Y Invertase yield= 61.40-34.60 (Inulin conc.)+18.73 (CSL conc.)+3.61 (BSM conc.)-7.94 ($(NH_4)_2SO_4$) + 2.40 ($NaNO_3$)-13.17 (KH_2PO_4)-8.39 (pH)-1.07 (Inoculum size)-9.04 (Incubation period)-16.80 (Temperature degree)-11.83 (Agitation speed) Equation (4).

where Y is the predicted response.

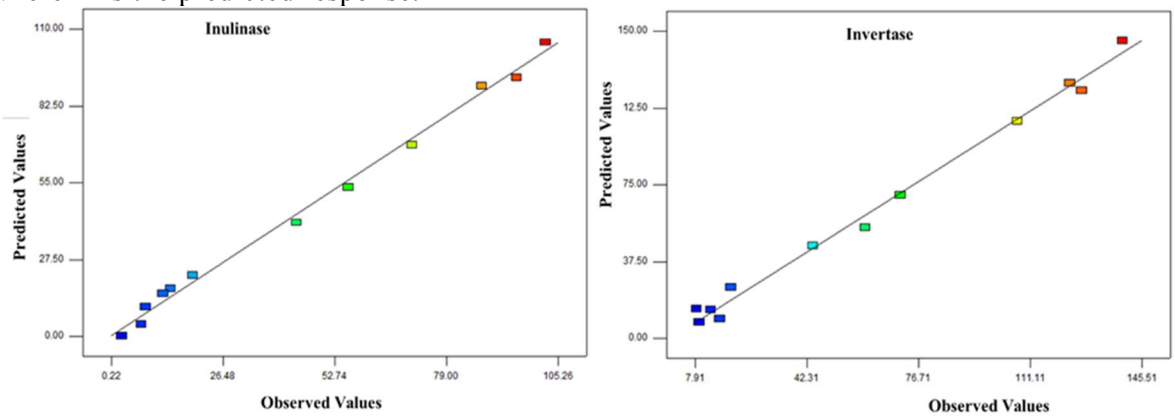

Fig. 3: Correlation of Predicted *vs.* observed values by the model for inulinase and invertase production by co-cultures of *A. niger and C. oleophila*.

In addition, Gong *et al.,* (2008); Bonciu *et al.,* (2011) reported that the highest inulinase and invertase activities from *Pichia guilliermondii* and *Rhizoctonia* spp. (coded I-PN4) were recorded at low temperature degree (with negative impact). Moreover, KH_2PO_4 had positive impact on inulinase and invertase production have been mentioned by Venkateshwar *et al.,* (2010); Bonciu *et al.,* (2011). Furthermore, Dinarvand *et al.*, (2017) showed that initial pH and temperature degree were significant effect on inulinase activity from *A. niger* ATCC 20611,while inoculum size and agitation speed were slightly lower effect. The increment in inulinase activity in presence of CSL may be due to the increase in fungal growth and interpreted on the basis that corn steep liquor serves not only as a nitrogen source but also as a source of growth factors which play an important role in enhancing the bacterial growth and enzyme production have been reported by Shady *et al.,* (2000); Ebeed and Abou-Taleb (2014).

From analysis model results, the points prediction of this model exhibited that the maximum production of inulinase and invertase under optimal experimental conditions was 103.2 and 140.1 U/ml, respectively.

Applications of microbial inulinase

Inulin hydrolysis

Results in Fig. 4 represented that % inulin hydrolysis by mixed crude inulinase produced from co-cultures *A. niger* and *C. oleophila* was increased gradually during reaction time and reached to maximum peak (75 and 61% of fructose content) after 60 min on pure inulin and chicory inulin, respectively. Whereas the % fructose content was observed to be more constant with increasing the reaction time from 90 to 120 min. These results are in trend line with those of Saber and El-Naggar (2009) reported that % inulin hydrolysis by *A. tamarii* AR-IN9 inulinase was reached to 55.11, 66.81, 67.55 and 71.64% on chicory roots, pure inulin, dahlia tubers and Jerusalem artichoke after 120 min of reaction time, respectively. moreover, Sirisansaneeyakul *et al.* (2007)found that mixed inulinase with ratio of 5:1 from *A. niger* TISTR 3570 and *C. guilliermondii* TISTR 5844.attained 28% inulin hydrolysis of Jerusalem artichoke tubers with 25 h reaction. In addition, Cruz *et al.* (1998) suggested that 72% chicory inulin hydrolysis was attained after 30 of reaction time by *A. niger*inulinase.

So, mixed inulinase converted inulin in plant residues which beneficial environmental effect.

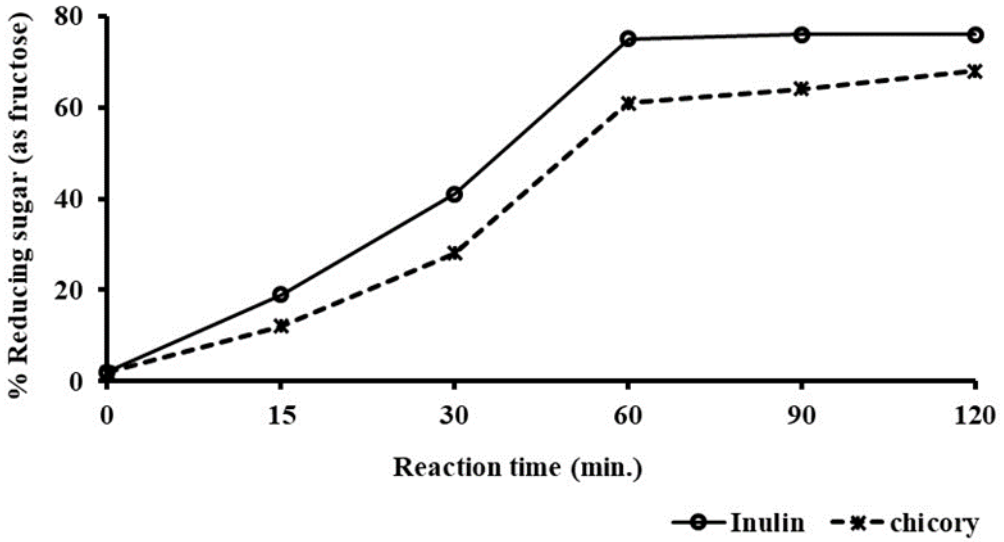

Fig. 4: % inulin hydrolysis by mixed *A. niger* and *C. oleophila* crude inulinas during various reaction time.

Ethanol production by A. niger and C. oleophila inulinase

In this an experiment, the ethanol was produced using 3 processes was as follows direct conversion (DC) of inulin into ethanol by mixed cultures *A. niger* and *C. oleophila*, separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) in both processes of SHF and SSF, inulin hydrolysis was fermented by *S. cerevisiae* to ethanol. Results in Fig. 5 indicated that mixed cultures of *A. niger* and *C. oleophila* were fermented inulin directly to ethanol in productive medium, and produces low value of ethanol reached 2.83 g/L with productivity was 1.4 g/L/d (DC process), it might be due to poor ability of *C. oleophila* to ferment inulin to ethanol, which it had similar with *K. marxianus* capable to produce inulinase and ethanol in fermented medium directly, but the amount of ethanol was low compared with the amount produced by *S. cerevisiae* have been stated by Chi *et al.* (2011). Whereas SSF and SHF processes produced high ethanol amounts (7.36 and 16.60 g/L) and productivity (3.7 and 7.8 g/L/d) were higher significant than produced by DC process. Furthermore, SSF process attained 2.12 and 5.51times higher significant production of ethanol from inulin than SHF and DC processes, respectively. It might be due to the reducing sugar was accumulated in SHF process resulted from hydrolysis which inhibits or decrease enzyme activity have been suggested by Margeot *et al.* (2009). Neagu and Bahrim (2012)observed that ethanol production from pure inulin and Jerusalem artichoke tubers by SSF method significantly increased was approximately 3 and 14 times more than production by SHF method, respectively. Likewise, Suttikul *et al.* (2016)

revealed that SSF process was most efficient technique for ethanol production from sugarcane trash which was 16.3% higher than of SHF process.

Therefore, SSF technique was selected for ethanol production from inulin in next studies.

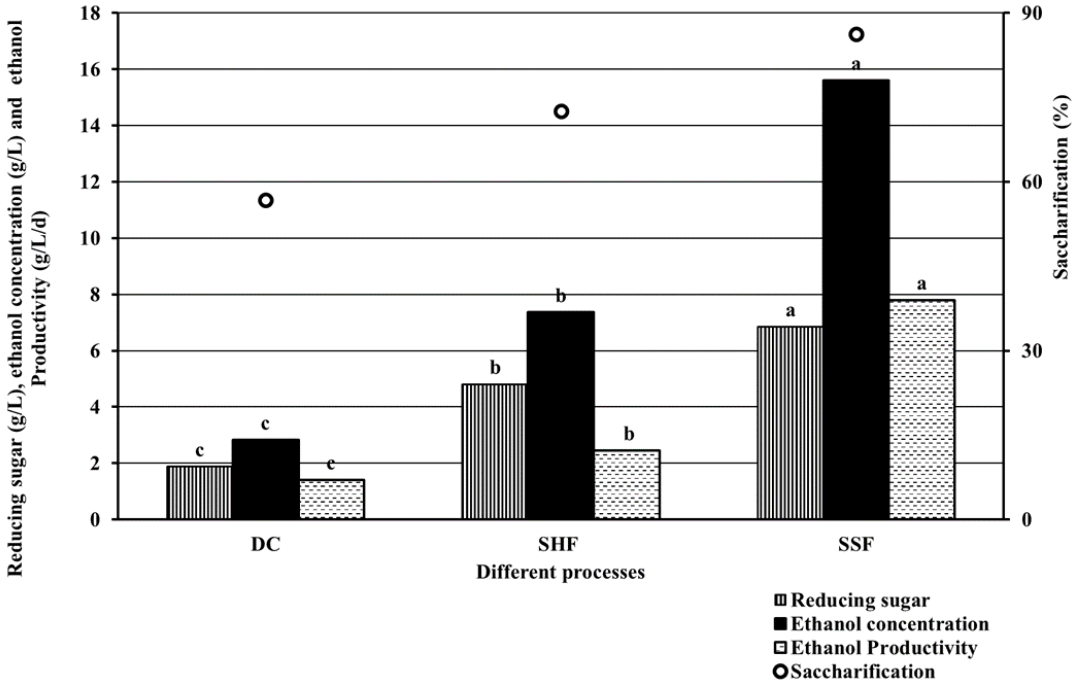

Fig. 5: Inulin saccharification and ethanol production from inulin by mixed *A. niger* and *C. oleophila* inulinase using different processes.

DC= direct conversion of inulin into ethanol by mixed cultures *A. niger* and *C. oleophila*, SHF = separate hydrolysis and fermentation and SSF= simultaneous saccharification and fermentation. Values presented in the same column have the same letter, do not significantly differ from every different at 5 % level.

Conclusion

In the present study, two strains of *A. niger* and *C. oleophila* was selected for degradation of inulin chicory root by production of inulinase as well as invertase. The applicability of statistical Taguchi orthogonal array approach demonstrated beneficial for optimization of fermentation parameters for inulinase and invertase production from mixed cultures of *A. niger* and *C. oleophila*. The predicted values of inulinase (103.2 U/ml and invertase (140.1 U/ml) were in good agreement with the experimental values been 102.3 and 139.5 U/ml, respectively. The optimized medium was consisted of (g/L): 15, inulin; 1.5, CSL; 0.5,$(NH_4)_2SO_4$; 3, KH_2PO_4 and adjusted pH to 5.5 then the medium was inoculated with 4% inoculum size and incubated at 30°C and 120 rpm for 2 days. *A. niger* and *C. oleophila* inulinase was applied to saccharification of inulin chicory root for production of fructose. In addition to, the production of ethanol from fermented inulin by *S. cerevisiae* using SHF and SSF operations. SSF was the best process for bioethanol production compared to SHF. bioethanol is an ecofriendly.

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