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An Innovative Synergism Between *Aspergillus oryzae* and *Azotobacter chroococcum* for Bioconversion of Cellulosic Biomass into Organic Acids under Restricted Nutritional Conditions Using Multi-Response Surface Optimization

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ABSTRACT
The ready available biomass of Rice Straw (RS) and Faba Bean Straw (FBS) cause serious environmental problems to several countries. A novel synergistic technique was applied using *Aspergillus oryzae* S2 in association with *Azotobacter chroococcum* to mediate the bioconversion of RS, FBS and Rock Phosphate (RP) into organic acids. Six-days of fermentation were optimal for improving C/N ratio and biodegradation of Fermented Biomass (FM) without previous pretreatment. Box-Behnken factorial design, with three independent variables: RP, $(NH_4)_2SO_4$ and FBS has been adopted for multi-response surface optimization for biodegradation of FM, using the Desirability function (D) approach. Solving the obtained equation model, determines the optimum levels of the independent variables at 120 mg P from RP, 10.39 mg $(NH_4)_2SO_4$ and 0.92 g FBS per 10 g RS. These levels were validated, the responses were 37.11% RDW and 17.13, 4.70 and 12.43 mg g^{-1} FM for total, soluble and insoluble sugars, respectively, with D value of 0.892, indicating the efficacy of biodegradation process. The HPLC analysis of the filtrate of the optimized FM contained (mg g^{-1} FM) 15.270 citric and 13.715 succinic acids as the major acids, beside minor amounts of ascorbic, oxalic, itaconic and maleic acids. The fungal strain was molecularly identified as *A. oryzae* S2 (KJ487973).

Key words: Solid-state bioconversion, organic acids, rice straw, multi-response surface optimization, desirability function

INTRODUCTION

The government's strategy is to maximize food production, according to the United Nations estimation by the year 2020; the world's rice harvest must increase to 840 million metric tons per year to meet the demands (FAO., 1987). Consequently, more Rice Straw (RS) will be generated as biomass residue during the harvesting process. Burning RS have global environmental risk implications. If it is not being used alternatively, it will create environmental problems. Faba Bean Straw (FBS) is another biomass residue with lower environmental impact and higher nitrogen content than RS, it contains reasonable amount of nitrogen. So, mixing both RS and FBS may introduce some balance in the carbon to nitrogen (C/N) ratio of the fermented biomass. The bioconversion of such organic biomass is depending not only on the environment for the process but also the nature of the input material, so, it is recommended to adjust the C/N ratio of

the fermented materials around 20, out of this range the rate of fermentation and/or microbial growth slow down (FAO., 1987; Lasaridi and Stentiford, 1998), leading to failure of decomposition process. Rock Phosphate (RP) is a general term that refers to rock with high concentration of phosphate minerals, most commonly of the apatite group, it is the major resource mined to produce phosphate fertilizers for the agriculture sector. The mineralization of RP by microorganisms is mainly induced by the secretion of organic acids (Saber *et al*., 2010), this is the major advantage of composting RP.

Response Surface Methodology (RSM) is useful statistical mathematical method used for analyzing, improving and optimizing the experiments by finding the analytical relationship between input and output variables considered in the fermentation medium and culture conditions. RSM also has the ability to produce an approximate function using a smaller amount of data and fewer numbers of experimental runs required to determine optimal conditions (Rodrigues *et al*., 2013). Most previous applications based on RSM have only dealt with a single-response problem, although several input variables having effect on several output responses usually faces scientists. Simultaneous optimization of the correlated response variables has become more important in complex systems. The object of multi-response optimization is to determine conditions of the independent variables that lead to optimal or nearly optimal values of the response variables simultaneously, in this respect, the desirability function approach is one of the most widely used methods for the optimization of multiple response problems (Costa *et al*., 2011; Hejazi *et al*., 2011).

The biosynthesis of organic acids by filamentous fungi like those of the genus *Aspergillus* are often used for industrial production of organic acids (Saber *et al*., 2010). However, to the best of our knowledge, no previous report about using mixture of inocula containing both fungi for degradation of biomass and bacteria such as the non-symbiotic N-fixing *Azotobacter chroococcum* as natural and cheap N-source, in order to adjust the C/N ratio of fermented medium, to optimize the bioconversion process. Organic acids are important group of chemicals that are extensively used in a variety of purposes, such as food preservatives, pH adjuster, sweetness enhancer, leavening agents, the pharmaceuticals and agricultural industries and stabilizers (Majumder *et al*., 2010; Rodrigues *et al*., 2013). Therefore, it is of great importance to find out cheap alternative sources for production process, however, cellulosic biomass such as the straw of rice and faba bean in addition to rock phosphate are good candidates especially when fermented by the suitable microorganisms. Two groups of organic acids are defined; however, both of them are delivered from glucose, those with a long biosynthetic pathway from glucose, involving much of the glycolytic pathway and the tricarboxylic acid cycle and those acids with a short pathway, essentially a biotransformation of glucose (Mattey, 1992). Since, glucose plays the central role in the bioconversion process, rice straw, as indirect source of glucose is good alternative which contains abundant amount of glucose, unfortunately in complex form (cellulose and lignin). Bioconversion of such complex materials into organic acids by the synergism between fungi and bacteria is economically feasible and that is the main target of the present study.

MATERIALS AND METHODS

Microorganisms, rock phosphate and straw: The certified and authentic strain of non-symbiotic nitrogen fixing bacterium, *Azotobacter chroococcum* with considerable nitrogenase activity was kindly provided by Biofertilizers Unit, Microbiology Department, Research Institute of Soils, Water and Environment, Agricultural Research Center, Giza, Egypt. This strain is used for the commercial production of N-fixing biofertilizers at this unit. *Aspergillus* sp. S2 was previously isolated as cellulolytic and complex phosphate solubilizing fungus (Saber *et al*., 2010) in Microbial Activity Unit of the same previously mentioned Microbiology Department. On the base of cultural properties, morphological and microscopic characteristics, the strain was identified as *Aspergillus oryzae* S2. RPcontaining 7.97% P, were kindly obtained from Research Institute of Soils, Water and Environment, Agricultural Research Center, Giza, Egypt. Rice Straw (RS) and Faba Bean Straw (FBS) were collected from Tag El-ezz Research Station, Dakahlia, Egypt.

Inocula preparation: *Azotobacter chroococcum* was grown in modified Asby's medium (Abd-El-Malek and Ishac, 1968) with shaking at 28°C for 48 h, followed by centrifugation at 5000 rpm to separate the bacterial cells which resuspended in sterilized water to obtain 10^8 cells mL^{-1}, approximately. *A. oryzae* S2 was inoculated on potato dextrose agar plates and incubated at 28°C for 7 days. The fungal colonies were covered with 10 mL of sterile distilled water and suspensions was made by gently probing the surface with the tip of a Pasteur pipette, the spore suspension was adjust to about 10^7 spore mL^{-1}. Before dual inoculation, compatibility between both microbes was checked by the antagonism test.

Design of Box-Behnken and fermentation procedure: A Box-Behnken factorial design (Box and Behnken, 1960), with 5 center points and 17 experimental runs, was used for the multi-response surface optimization for bioconversion of Fermented Biomass (FM). The fermentation was carried out using 10 g of RS as solid state in 500 mL Erlenmeyer flasks. Three independent variable were studied, each at three coded levels -1, 0 and +1 for low, middle and high concentrations, respectively. The independent variables were RP (X_1), $(NH_4)_2SO_4$ (X_2) and FBS (X_3). The levels of each independent

Table 1: Box-Behnken design using three tested independent variables with five center points showing the codded and actual values per 10 g RS, as well as the response variables of bioconversion process as a function of synergism between *Aspergillus oryzae* and *Azotobacter chroococcum*

	Tested independent variable						Response variable				
	RP (mg P) (X_1)		$(NH_4)_2SO_4$ (mg N) (X_2)		FBS (g) (X_3)				Sugar (mg g^{-1} FM)		
Run	Code	Actual	Code	Actual	Code	Actual	RDW (%)	TA (meq. KOH g^{-1} FM)	Total	Soluble	Insoluble
1	-1	20	-1	2.5	0	1.0	62.3	53.1	7.62	2.03	5.58
2	1	120	-1	2.5	0	1.0	50.0	26.5	9.54	2.93	6.61
3	-1	20	1	10.5	0	1.0	43.1	34.6	15.02	2.49	12.53
4	1	120	1	10.5	0	1.0	40.0	17.3	17.51	4.80	12.71
5	-1	20	0	6.5	-1	0.5	85.4	61.2	2.33	1.52	0.81
6	1	120	0	6.5	-1	0.5	47.7	35.3	11.79	3.39	8.40
7	-1	20	0	6.5	1	1.5	50.0	67.7	9.95	1.36	8.58
8	1	120	0	6.5	1	1.5	63.1	17.3	6.48	3.42	3.07
9	0	70	-1	2.5	-1	0.5	73.8	70.4	2.79	1.43	1.36
10	0	70	1	10.5	-1	0.5	55.6	26.5	7.78	2.95	4.82
11	0	70	-1	2.5	1	1.5	67.5	34.6	3.79	2.24	1.55
12	0	70	1	10.5	1	1.5	48.5	53.1	10.85	2.10	8.75
13[C]	0	70	0	6.5	0	1.0	57.4	61.2	10.25	5.91	4.34
14[C]	0	70	0	6.5	0	1.0	59.5	51.9	11.15	6.19	4.96
15[C]	0	70	0	6.5	0	1.0	62.3	61.2	12.00	6.74	5.26
16[C]	0	70	0	6.5	0	1.0	61.0	56.5	11.25	6.27	4.98
17[C]	0	70	0	6.5	0	1.0	60.8	56.5	11.25	6.65	4.60

[C]Center points

variable and the experimental design with respect to their coded and uncoded levels, as well as, the design matrix are presented in Table 1. For the three-factor system, the second order polynomial quadratic model was taken to be:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta 22 X_2^2 + \beta 33 X_3^2$$

where, **Y** is the predicted response, β_0 model constant; X_1, X_2 and X_3 independent variables; β_1, β_2 and β_3 are linear coefficients; β_{12}, β_{13} and β_{23} are cross product coefficients and β_{11}, β_{22} and β_{33} are the quadratic coefficients. For simultaneous optimization of the tested responses, a desirability function was specified for each response, the overall desirability of multi-response optimization is defined as the geometric mean of the desirability for each response. The statistical software package Design-Expert version 7 (Stat-Ease, Minneapolis, USA) was used for both constructing the design and analysis of experiments at probability (p) valve<0.05.

The solid stat fermentation medium was autoclaved at 121°C for 20 min. Inoculation was carried out using 5 % (v/w) from each of both *A. oryzae* S2 and *A. chroococcum*. The initial moisture content was adjusted to about 65%. The contents were mixed thoroughly and incubated at 30°C±1. The optimum incubation period was determined based on the time course profile of the bioconversion process of cellulosic biomass. The medium composition for testing the time course profile was prepared at the center points of the three factors according to Box-Behnken experimental design. After incubation, 100 mL of distilled water was added to each flask, shaken for 30 min on a rotary shaker at 150 rpm and filtered through Whatman No.1 filter paper. The residue of fermented biomass and resulted filtrate was used to evaluate the bioconversion process.

Evaluation of bioconversion process and assay of cellulase: Multi-parameters were used to evaluate the bioconversion process. The Residual Dry Weight (RDW) of the fermented biomass was determined after drying at 70°C to constant weight. The total nitrogen and organic matter were determined (AOAC., 1995), both of them were used to calculate the overall C/N ratio in the fermented biomass. The filtrate of FMwas analyzed for the Total Acidity (TA) by titration with KOH using phenolphthalein indicator. With respect to sugars in the filtrate of in the FM, the total sugars was determined with phenol sulphuric acid (Dubois *et al*., 1956) soluble sugar by the method of Upmeyer and Koller (1973) and the insoluble sugars was calculated by subtraction of soluble sugars from the total sugars. The amount of every sugar type present in the sample solution was calculated using the standard curve of glucose prepared in the same procedure and expressed as mg g^{-1} FM.

The overall cellulolytic activity was estimated by the assay of filter paperase activity (FPase). One milliliter of filtrate was incubated with 50 mg filter paper Whatman No. 1 (1.0 cm ×6.0 cm) in 1 mL of 0.2 mole acetate buffer (pH 4.8) at 50°C for 60 min. The reducing sugars released were determined by the dinitrosalycylic acid method (Miller, 1959) using glucose as a standard. One unit of FPase activity (U) corresponding to 1 μM of glucose equivalent released per minute per gram of fermented biomass under the experimental assay conditions.

Determination of organic acids by the high-performance liquid chromatography (HPLC): A fermentation batch,

based on the optimum condition of the bioconversion process, was carried out to profile the organic acid content in the extracted hydrolysate. Analysis was performed with HPLC (Agilent 1100HPLC system), using an Agilent ZORBAX Eclipse XDB -C18 column, 5 μm particle size column and 4.6×150 mm with a mobile phase of 5 mM phosphoric acid (K_2HPO_4, pH 2.8), a flow rate of 1 mL min^{-1} and a column temperature of 30°C. Injection volume of the samples used for HPLC analysis was 20 μL of the extract. Detection was by UV absorbance at 220 nm. The organic acids were quantified by reference to the peak areas and retention times obtained for the authentic standards for six organic acids (ascorbic, oxalic, maleic, itaconic, citric and succinic acids) in mobile phase. For citric and succinic acids, the concentrations of the aqueous standard solutions were 0.8 and 0.6 mg mL^{-1}, respectively, the other organic acids were analyzed at a concentration of 0.01 mg mL^{-1}.The results of organic acids were expressed as mg g^{-1} FM.

Isolation of genomic DNA, amplification and 18S rRNA sequencing: The sequencing of 18S rRNA was performed by Macrogen Korea Company Gasan-dong, Geumchen-gu, Seoul, Korea (http://www.macrogen.com). The genomic DNA was isolated by transferring fungal mycelium with a sterilized toothpick, suspended in 0.5 mL of sterilizes saline in a 1.5 mL centrifuge tube and centrifuged at 10000 rpm for 10 min. Then the supernatant was discarded and the pellet was resuspended in 0.5 mL of Insta Gene Matrix (Bio-Rad, USA), incubated at 56°C for 30 min and then heated (100°C for 10 min). After heating, supernatant can be used for PCR. The PCR amplification reaction was performed in a total volume of 100 μL which contained 1 μL DNA, 10 μL of 250 mM deoxyribonucleotide 5'-triphosphate (dNTP's), 10 μL PCR buffer, 3.5 μL 25 mM $MgCl_2$ and 0.5 μL *Taq* polymerase, 4 μL of 10 pmol (each), forwardprimer ITS1 (3'-TCCGTAGGTGAACCTGCGG-5') and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC -3') and water was added up to 100 μL. The PCR-apparatus was programmed to perform 5 min denaturation at 94°C, followed by 35 amplification cycles of 1 min at 94°C, 1 min of annealing at 55°C and 2 min of extension at 72°C, followed by 10 min final extension at 72°C. Unincorporated PCR primers and dNTPs were removed from PCR products using Montage PCR Clean up kit (Millipore). The PCR reaction mixture was then analyzed via 1% (w/v) agarose gel electrophoresis and the remaining mixture was purified using QIA quick PCR purification reagents (Qiagen, USA). The purified PCR product was sequenced in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with Ampli *Taq*-DNA polymerase (FS enzyme) (Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using the last mentioned PCR-primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and sequencing product was resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied Bio Systems, USA). The sequenced product was deposited in the Gen Bank database under accession number KJ487973. The 18S rRNA gene sequence of the strain was aligned with the corresponding 18S rRNA sequences of the type strains of representative members of the fungi retrieved from the GenBank, EMBL, DDBJ and PDB databases by using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al*., 1997) and the software package MEGA4 version 2.1 (Tamura *et al*., 2007) was used for multiple alignment and phylogenetic analysis. The phylogenetic tree was constructed via the neighbor-joining algorithm (Saitou and Nei, 1987) based on the 18S rRNA gene sequences of the strain and related organisms.

RESULTS

Before the dual inoculation by *Aspergillus* sp. S2 and *Azotobacter chroococcum*, antagonism was carried out on PDA plates to discover the possible antagonism between both microbes. Results obtained herein indicate no visible antagonism could be observed. On contrary, a clear compatibility had been confirmed. Hence, the dual inoculation was considered during the following bioconversion process. *Aspergillus* sp. S2 was identified as *Aspergillus oryzae* S2, based on cultural, morphological and microscopic characteristics.

Time course of microbial biodegradation of FM: The results (Fig. 1) of the periodic biodegradation process show that the hydrolysis of fermented biomass (RS, RP, $(NH_4)_2SO_4$ and FBS) was achieved earlier at the second day of the dual incubation of *A. oryzae* S2 and *Azotobacter chroococcum*. Maximum release of total (12.6 mg g^{-1} FM) and soluble (6.4 mg g^{-1} FM) sugars as well as FPase (11.1 Ug^{-1} FM) were observed after 6 days of incubation. After that, there was no additional degradation. Oppositely, there was continual reduction in the weight of FM and the C/N ratio with the time of incubation. However, 6 days of fermentation was taken as the ideal period for bioconversion process.

Optimization of bioconversion process by Box-Behnken design: Box-Behnken design was adopted to explain the interaction among three independent variables; viz., RP, $(NH_4)_2SO_4$ and FBS in the presence of RS, on the Solid-State Bioconversion (SSB) process, using the synergism between *A. oryzae* and *Azotobacter chroococcum*. The independent variables of SSB process were used at three different concentrations. The response variables (RDW, TA and the three types of released sugars) are presented in Table 1. The

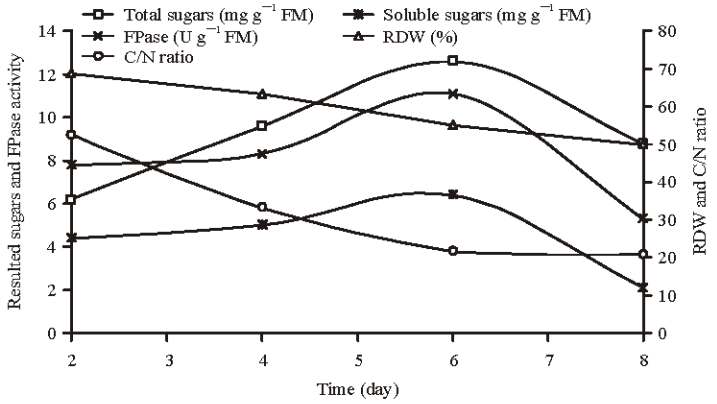

Fig. 1: Biodegradation of FM by the dual inoculation with *A. oryzae* S2 and *Azotobacter chroococcum* as a function of time

Table 2: Simple correlation coefficient (r) between every two responses based on results of box-behnken design

Parameters	RDW	Total acidity	Total sugars	Soluble sugars
Total acidity				
Pearson R	0.462^{NS}			
p-value	0.062			
Total sugars				
Pearson R	-0.846^{S}	-0.268^{NS}		
p-value	0.000	0.299		
Soluble sugars				
Pearson R	-0.121^{NS}	0.054^{NS}	0.513^{S}	
p-value	0.644	0.837	0.035	
Insoluble sugars				
Pearson R	-0.916^{S}	-0.343^{NS}	0.873^{S}	0.029^{NS}
p-value	0.000	0.178	0.000	0.911

^{S}Significant at p<0.05, ^{NS} Not significant

Pearson correlation coefficient (r) was estimated to measure the degree of relationship between each pairs of response variables (Table 2). The value of ranged between -1 and +1. If one variable tends to increase as the other decreases, then between the two variables is negative and vice versa for the positive R. Upon this base, the RDW was positively but non-significantly correlated with total acidity (R = 0.462^{NS}), whereas, negatively correlated with the different kinds of released sugars, the values of Pearson r recorded-0.846,-0.121^{NS} and -0.916 for total, soluble and insoluble sugars, respectively. Among the three kinds of sugars, only the total sugars is positively correlated with both soluble (r = 0.513) and insoluble (r = 0.873) ones, whereas there was no correlation between soluble and insoluble sugars. The TA, on the other hand did not show significant correlation with any of the tested variables.

The data of Box-Behnken design were fitted with a second order polynomial function. The analysis of variance (Table 3) indicates that the overall model terms of the various responses are significant, where the p-values did not exceed than 0.0004 for any of the tested responses. Contrarily, the lack of fit error of p-value did not reach the level of significant, in which the p-value for any of the tested parameters was ≥0.160. The coefficient of determination (R^2), the adjusted R^2 and predicted R^2 values were significant for all the tested responses. The values of all kinds of R^2 are in reasonable agreement, recording ≥0.9, except the predicted R^2 of the RDW, TA and total sugars, being 0.801, 0.548 and 0.863, in sequence. However, the higher the R^2 the more accurate of the mode. In addition, the models have high adequate precision values of >14. Finally, the predicted residual sum of squares (PRESS) values were relatively low, except PRESS of TA. All the previous values lead to the conclusion that the model could be effectively used to measure the particular model fits at each point in the design, indicating the validity of the data of the different responses, TA is the only exception. The three independent variables (X_1, X_2 and X_3) were further statistically analyzed for single, interaction and cubic effect on the different response parameters, in this respect, the single effect of the model terms of X_1 (RP) and X_2 ($(NH_4)_2SO_4$) as well as the cubic effect of X_1^2 (RP^2) reached the level of significant with all the tested responses. Contrarily, the model term interaction of X_1X_2 (RP and $(NH_4)_2SO_4$) was not significant with any of the tested response variables. The other terms vary between significant and non-significant based on the nature of the independent variable.

Multi-response optimization by desirability function and validation: Since, optimization of multi-responses individually may yield different and conflicting factor settings; the optimization was carried based on total desirability function (D) of multi-response surface, in which, each of individual response is transformed into a desirability value (d) and the D is then computed as the geometric mean of the different d. The D value lies between 0 and 1, as the response approaches the target, the D value becomes closer to 1. To calculate D, the RDW was set to be minimized and the three kinds of sugars to be maximized, whereas TA was removed since its low predicted R^2 (0.548) failed to provide valid predictive ability. Three-dimensional surface response plots of D (Fig. 2) were generated by varying two independent variables within the experimental range and holding the other constant at the central point. Solving this multi-response optimization problem led to obtain the levels of RP at 120 as

Table 3: Analysis of variance showing the p-valve and model evaluation of the response surface quadratic model for the different response variables of FM as a function of dual inoculation with *Aspergillus oryzae* and *Azotobacter chroococcum*

	Response variable				
			Sugar (mg g^{-1} FM)		
Source of variation	RDW (%)	TA (meq. KOH g^{-1} FM)	Total	Soluble	Insoluble
Model evaluation					
Model					
F-value	41.34^{S}	19.02^{S}	55.19^{S}	74.81^{S}	89.070^{S}
p-value	$<0.0001^{S}$	0.0004^{S}	$<0.0001^{S}$	$<0.0001^{S}$	$<0.0001^{S}$
Lack of fit					
F-value	2.34^{NS}	2.96^{NS}	1.79^{NS}	0.50^{NS}	2.970^{NS}
p-value	0.214^{NS}	0.161^{NS}	0.288^{NS}	0.700^{NS}	0.160^{NS}
R^2	0.982	0.961	0.986	0.990	0.991
Adjusted R^2	0.958	0.910	0.968	0.976	0.980
Predicted R^2	0.801	0.548	0.863	0.943	0.900
Adequate precision	25.54	14.29	27.08	21.64	31.510
PRESS	407.85	2211.33	35.81	3.54	19.280
p-value					
Single					
X_1	0.0005^{S}	$<0.0001^{S}$	0.0014^{S}	$<0.0001^{S}$	0.0492^{S}
X_2	$<0.0001^{S}$	0.0089^{S}	$<0.0001^{S}$	0.0034^{S}	$<0.0001^{S}$
X_3	0.0014^{S}	0.2052^{NS}	0.0167^{S}	0.8355^{NS}	0.0021^{S}
Interaction					
X_1X_2	0.0877^{NS}	0.4074^{NS}	0.7056^{NS}	0.0528^{NS}	0.4174^{NS}
X_1X_3	$<0.0001^{S}$	0.0517^{NS}	$<0.0001^{S}$	0.7694^{NS}	$<0.0001^{S}$
X_2X_3	0.8608^{NS}	0.0006^{S}	0.1945^{NS}	0.0288^{S}	0.0066^{S}
Cubic					
X_{12}	0.0017^{S}	0.0016^{S}	0.0080^{S}	$<0.0001^{S}$	$<0.0001^{S}$
X_{22}	0.0014^{S}	0.0023^{S}	0.8976^{NS}	$<0.0001^{S}$	0.0002^{S}
X_{32}	0.0005^{S}	0.8248^{NS}	$<0.0001^{S}$	$<0.0001^{S}$	$<0.0001^{S}$

^{S}Significant at $p<0.05$, ^{NS} Not significant

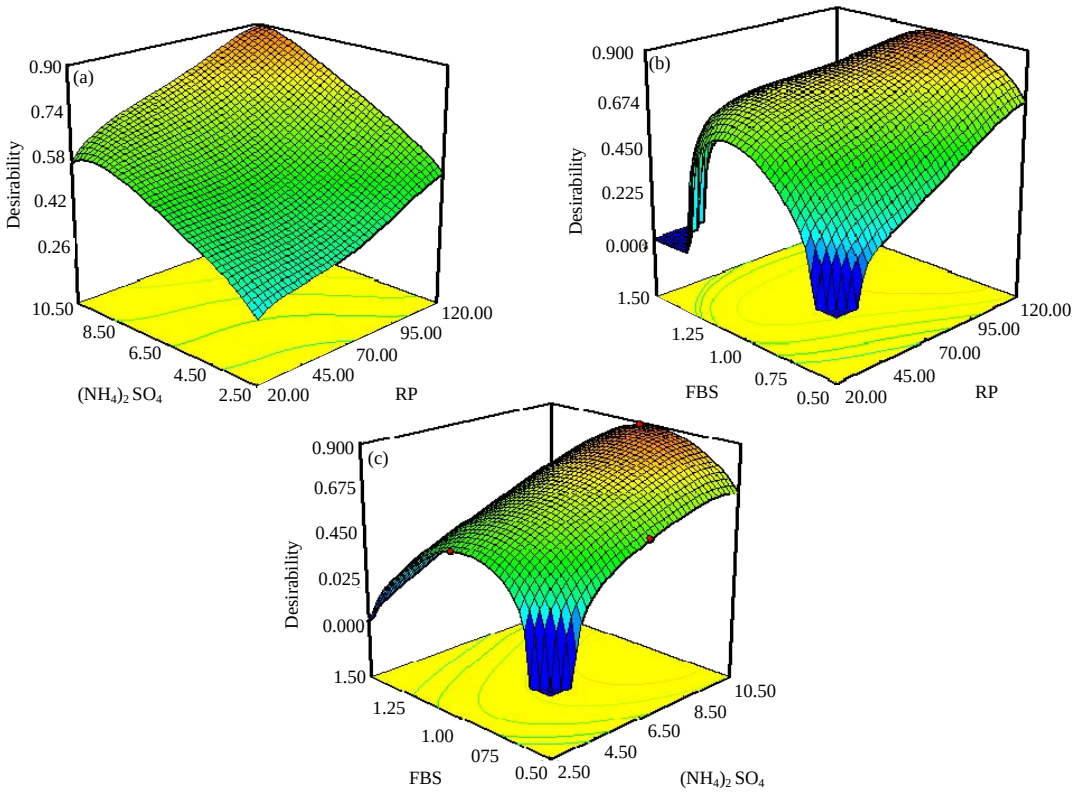

Fig. 2(a-c): Three-dimension plots showing the correlative effect of the tested variables on the overall desirability function in which (a) RP and $(NH_4)_2SO_4$ at hold value of FBS = 0.92 g per 10 g RS, (b) RP and FBS at hold value of $(NH_4)_2SO_4$ = 10.39 as mg N per 10 g RS and (c) $(NH_4)_2SO_4$ and FBS at hold value of RP = 120 as mg P per 10 g RS

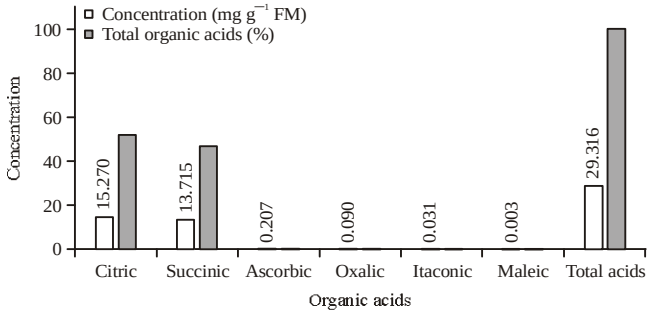

Fig. 3: Organic acids detected during SSB of FM as a function of synergism between *A. oryzae* and Azotobacter Chroococcum

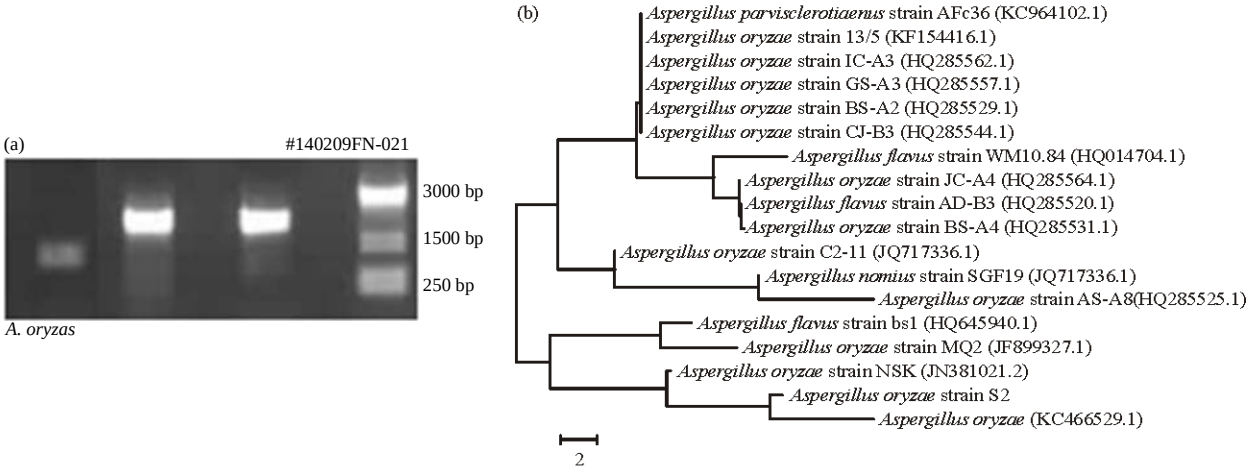

Fig. 4(a-b): Molecular identification of *A. oryzae* strain S2 showing (a) Agarose gel electrophoresis of PCR product band of the amplified 18S rRNA fragment and (b) Phylogenetic tree obtained by neighbor-joining analysis of 18S rRNA gene (partial), internal transcribed spacer 1, internal transcribed spacer, showing the position of *A. oryzae* strain S2 within the genus *Aspergillus*. Gen Bank sequence accession numbers are indicated in parentheses after the strain names. Bar, 2 substitution per nucleotide position

mg P, $(NH_4)_2SO_4$ at 10.39 mg and FBS at 0.92 g per 10 g RS, as solid state. The theoretical values of the response variables were 37.21% RDW and 17.38, 4.85 and 12.53 mg g^{-1} FM for total, soluble and insoluble sugars, respectively. At these levels, the D recorded 0.892. The previous calculated data was experimentally validated; the obtained Responses±Standard deviation recorded 37.11±1.20% RDW and 17.13±0.88, 4.70±0.56 and 12.43±0.455 mg g^{-1} FM for total, soluble and insoluble sugars, respectively.

Organic acids content in the filtrate of FM: The previous evidences for the efficient bioconversion of FM into sugars as well as the acidic nature of the hydrolysate, represented by TA, encourage the differentiation of organic acids that may be formed as a result of synergistic action of both *A. oryzae* and *Azotobacter chroococcum*. The production of organic acids was performed on a medium composition based on the previous validation test; the incubation period lasted for 6 days. Six organic acids in the filtrate of FM were identified and quantified using HPLC (Fig. 3) The total formed organic acids reached to 29.316 mg g^{-1} FM. Citric (15.270 mg g^{-1} FM) and succinic (13.715 mg g^{-1} FM) acids were the major acids detected, being 98.87% of total organic acids. The other organic acids detected in descending order were ascorbic, 0.207 >oxalic, 0.090 >itaconic, 0.031 >maleic, 0.004 mg g^{-1} FM. These organic acids were detected in negligible amount (1.13%), compared to citric and succinic acids.

Identification and phylogenetic position of the isolate S2: The molecular identification of cellulolytic RP-solubilizing *A. oryzae* S2 was done based on 18S rRNA gene sequencing. The obtained sequence of amplified 18S rRNA fragment on agarose gel (Fig. 4) showed similarity of the target sequence to the closely related fungi sequences to *A. oryzae*. The sequence was submitted to the Gene Bank under the accession number KJ487973 and aligned with other 18S rRNA sequences of representative *Aspergillus* species retrieved from the Gen Bank, EMBL and DDBJ databases by using BLAST searches showed 81-83% homology with that of

many species of the genus *Aspergillus*. The phylogenetic relationship between strain S2 and other related fungi, based on 18S rRNA gene sequencing of members of the genus *Aspergillus* was constructed (Fig. 4) according to the method of neighbor-joining. The tree shows the close phylogenetic association of strain S2 with certain other *Aspergillus* species. Phylogenetic analysis indicated that the strain S2 consistently falls into a clade together with *A. oryzae* strain NSK (JN381021.2) and *A. oryzae* (KC466529.1). Therefore, it is proposed that strain S2 should be included in the genus *Aspergillus* as *A.* oryzae strain S2 (KJ487973).

DISCUSSION

Nature is abundant with many kinds of relationships among microorganism, of them; the synergism is a kind of complimentary one, in which two or more microbes have one target. Where, one of microbes performs a specific role or function, then the second microbe drives the mission to its final destination. That is the main idea of the present work. Herein, the role of *Azotobacter chroococcum* was to mediate the fermentation conditions and the role of *A. oryzae* S2 was to complete the fermentation to produce organic acids, some overlap may be occurred by exchanging the role between microbes but everyone know well its main role.

Aspergillus sp. S2 was isolated in previous study (Saber *et al*., 2010) as cellulolytic and rock phosphate solubilizer. It was initially, identified as *A. oryzae* S2 based on cultural properties, morphological and microscopic characteristics. The dual inoculation with *Azotobacter chroococcum* is to augment the required nitrogen during the fermentation process; this expected to adjust the C/N ratio which helps the microbial growth and the biodegradation of the fermented biomass. These unique criteria for each microorganism and in the same time, their compatible in growth without antagonism, introduce complementary and integrated functions for the cheap growth medium which is poor from the nutritional point of view. The SSB was the suitable fermentation technique for such medium. The attraction of SSB comes from its simplicity and closeness to the natural way of life for many microorganisms, high volumetric productivity and as an alternative in preventing environmental pollution. Moreover, SSB is another approach to reduce the cost by using the lignocellulosic materials as substrates rather than expensive pure cellulose (Khan *et al*., 2007). RS and FBS are good candidates for such bioconversion; both of them were used in this study without pretreatment which restricts the fermentation conditions under the current poor medium.

Time course of SSB system of biomass using RS as a solid substrate was carried out taking into account the RDW, FPase activity and sugars (total and soluble) as indicators for the bioconversion process, as well as, C/N ratio as another indicator for the improvement of medium composition. The C/N ratio tend to decrease gradually with time, this is due to the gaseous loss of carbon as CO_2, while the nitrogen remained more tightly bound in organic combination as long as the C/N ratio is wide (Kaloosh, 1994). This in turn helps keeping the C/N ratio at suitable level (about 20) that suits the microbial growth and bioconversion process (FAO., 1987). When C/N rises, the rate of fermentation slows down due to the limitation of nitrogen. On the other hand, when the ratio of C/N drops greatly, excess nitrogen is lost to the air as ammonia and there is a rise in pH level which may be toxic to some microorganisms (Lasaridi and Stentiford, 1998). The cellulase activity of filter paper substrate successfully maximized in the filtrate of the dual culture at the 6th day then decreased with the fermentation period finishes. This decreasing trend of FPase activity which estimates the overall cellulolytic activity, was due to the reduction of nutrients supplied in the substrate and the accumulative effect of cellobiose, the dimer of glucose known to inhibit both endoglucanase and glucosidase (Howell and Mangat, 1978). The total and soluble sugars occupied the same path of FPase, they increased gradually up to the 6th day, then decreased sharply afterwards, suggesting their dependency on the activity of cellulases, as well as indicating their continual bioconversion to other compounds, where there was no additional accumulation of sugars when cellulase activity declines. However, 6 days is reasonably accepted because of the poverty of the fermentation medium and no any pretreatment was carried on the FM, these two points make the SSB process difficult on the microbes. However, 6 days was the optimum fermentation period for citric acid recovery from sugarcane molasses using *A. niger* (Farooq *et al*., 2013).

For multi-optimization of the different responses, the statistical design Box-Behnkenwas applied which guarantees several advantages over the one variable at a time approach for optimizing microbial products which may be effective in some situations but is inadequate as it is a time consuming process and it further neglects the interaction between variables and does not guarantee attaining optimal points (Myers and Montgomery, 1995). The statistical designs provide efficient and effective coverage of the experimental space with few measurements, it can be adopted at various phases of an optimization strategy, such as for screening experiments or looking for the optimal conditions for targeted of one or more response (Box and Behnken, 1960; Dehghani and Nekahi, 2010). Although, the use of RSM in biotechnological processes is gaining immense importance for the optimization of several bioprocesses, the multi-response problems have received only limited attention (Hejazi *et al*., 2011). The interaction among the three independent variables (RP, $(NH_4)_2$ SO_4 and FBS) was investigated using Box-Behnken design which is experimental designs for RSM.

Data of the different responses of Box-Behnken design were first subjected to correlation analysis, in which the RDWof FM was found to be negatively correlated with the different kinds of released sugars; this could be explained by the bioconversion of FM into the three kinds of sugars. Also, indicate the efficacy of the biodegradation process, however, the secreted FPase previously detected in the filtrate of the FM

plays important role in this respect, where sugars, especially the soluble one, is the target product of cellulase enzymes. The total sugars is positively correlated with both soluble and insoluble sugars, because total sugars are the sum of them. Whereas there was no correlation between soluble and insoluble sugars, this may be back to the kind of cellulase enzyme secreted. Although the evidences for the acidity nature of the products indicated by the TA, there was no significant correlation between TA and any of the other tested variables. For the first round evaluation of the models of the various responses of Box-Behnken matrix, the data were fitted with a second order polynomial function based on ANOVA. The accuracy of the models was confirmed and all the models of the tested responses were significant which are represented by both high F-values and small p-values(<0.05). The lack of fitswere not significant relative to the pure error, being small F-values and high p-values(>0.05). The lack of fit is an indication for the appropriateness of the data, as fitting of model is required and reflicts a good parameter. High F-value of the model and non-significant lack of fit indicate the adequate fitness of the model. The multiple hypothesis; F-test is usually used for model evaluation, to determine if the variances among the means of populations are significantly different or equal. The F-test is applied assuming no variations between the variances (null hypothesis, H_0). The alternative hypothesis (H_1) is the one that would replace if the variances are varied from each other (rejection of H_0). It is important to know that F-value determines the p-value and all hypothesis tests, for practical purposes, ultimately use p-value to decide to either accept or reject H_0. A small p-value (<0.05) indicates strong evidence to reject H_0, whereas, large p-value (≥ 0.05) is an evidence to accept H_0. Therefore, p-value is considered a guideline to ignore data that does not reach a specified significance level. The present data showed strong evidence to reject H_0, i.e., there are significant variation among the tested means of the various responses (RDW, TA and the three kinds of sugars).

The second round evaluation of the different models is depending on the three kinds of R^2 which should be positive with value of ≥ 0.75 and close to each other. The values of R^2 of the various responses were more than 0.96, i.e., the model can explain more than 96% of variation for any of the different responses. The adjusted R^2 ranged from 0.910 for TA to 0.980 for the insoluble sugars and the predicted R^2 ranged from 0.548 for TA to 0.943 for the soluble sugars. The values of adjusted and predicted R^2measure how well the fitness of the data, This can help selection the model with the best fit. Predicted R^2 can prevent overfitting by providing valid model predictions for the new observations and is more useful than adjusted R^2 for comparing models. However, the higher of their values, the more aptness of the model and the more accuracy of the relationships between the tested variables and the response. The models of RDWof FM and the three kinds of the resulted sugars, reported herein, are ideal in this respect. However, TA is the only exclusion of our data.The values of adequate precision occupied a range from 14.29-31.51, suggesting that the model of each response can be used to navigate the design space. Adequate precision is an index of the signal to noise ratio, its value at >4 is an essential prerequisite for a model to be a good fit. Assessing the model's predictive ability of PRESS is additional indicator for model evaluation which is used to calculate the predicted R^2 which is generally more intuitive to interpret. Together, these statistics can help prevent overfitting the model because it is calculated using observations not included in model estimation. Generally, the smaller the PRESS value, the better the model's predictive ability.

Within each response, the single effect of the model terms of X_1 (RP) and X_2 ($(NH_4)_2SO_4$) as well as the cubic effect of X_1^2 (RP^2) reached the level of significant with all the tested responses, contrarily, the model term interaction of X_1X_2 (RP and $(NH_4)_2SO_4$) was not significant with any of the tested responses. The other terms varied between significant and non-significant based on the tested variable. These data reflect the importance of RP as strong inducer for the bioconversion of biomass with final products having acidic nature, i.e., organic acids. The presence of RP plays indirect role towards the formation of organic acids, because RP is the only complex non-soluble source of phosphorus. To obtain their needs from soluble phosphorus, microorganisms solubilize RP to get soluble phosphorus for their growth and multiplication. Solubilization of RP is mediated by the secretion of phytase and organic acids by the microorganism (El-Naggar and El-Hersh, 2011; Saber *et al*., 2010). The secretion of organic acids is associated with reduction in pH; this situation was noticed in our data, wherein, the TA, even at low values, is an indication for the presence of acidic substances. $(NH_4)_2SO_4$ is simple nitrogen source that is required, nearly in all growth stages of microorganism, the small amount encourage the growth of fungi at the initial stage of bioconversion process. Additionally, N-fixation is reduced in the presence of high N-content in the growth medium but low amount of N decreases the lag phase and generation time and thus fermentation time (Tejera *et al*., 2005), that is why small amount of $(NH_4)_2SO_4$ was used in the present work. Because of its high content of complex N, FBS acts as a source of continual N (Bond *et al*., 1985) which may also help adjusting C/N ratio along the bioconversion period, by its slow release, compared with $(NH_4)_2SO_4$. It is also acts as a source of biologically convertible biomass as in the case of RS. However, the three kinds of N-sources (FBS, $(NH_4)_2SO_4$ and *Azotobacter chroococcum*) may introduce complimentary balance for N supplementation during bioconversion process. The second round evaluation of statistical analysis of the models of the different responses ends with the conclusion of omitting TA from multi-response optimization, using the desirability function. Although its model was significant. This exclusion because of its lower value of predicted R^2(0.548); this means low precision and poor predictive ability of this model. The other responses confirmed satisfactory adjustments of the quadratic models to the experimental data; that is why RDW and the three types of sugars were included in the multi-response optimization, using the desirability function.

Validation of the bioconversion process of the fermented biomass after statistical optimization showed that the experimentally determined values were in close agreement with the statistically predicted ones, confirming the model's authenticity, the multi-optimization was carried out based on D which recorded 0.892, this value is reasonably high. One useful and widely used approach to optimize multiple responses is to utilize the D, the simultaneous optimization technique popularized by Derringer and Suich (1980). Their common approach is to first transform each response into an individual d that varies over a range of between 0 and 1, it is proportional to the priority given to the response variable and increases as the corresponding response value becomes more desirable. Depending on whether a particular response is to be maximized, minimized, or assigned to a target value, different desirability functions can be used, only by doing so, one can achieve the ideal balance among the desired response levels (Aksezer, 2008). The method finds operating conditions of the different tested factors that provide the most desirable response values of the tested parameters, by combining the individual responses into a composite function followed by its optimization (Costa *et al*., 2011).

At this point, there were reasonable evidences for the possible biosynthesis of organic acids, these evidences include the formation of soluble and insoluble sugars, the precursor of organic acid formation, the presence acidic substances, indicated by TA and the reduction in FM represented by RDW. This in turn, encouraged the differentiation of the organic acids profile in the filtrate of the FM. Many methods are used for analyses of organic acids, however, because of simplicity, accuracy and speed of analysis, the HPLC technique is preferred method, by which two major organic acids (citric and succinic acids) were synthesized in high concentration compared with the other detected ones, both were represented by 98.87% of total organic acids. During microbial activity on various substrates under different nutritional conditions, organic acids are formed as metabolites, for example, citric acids is the major acidic metabolite produced by *Penicillium bilaii* which is promoted under nitrogen-limited conditions. While oxalic acid production is promoted under carbon-limited conditions (Cunningham and Kuiack, 1992). The detected organic acids herein were biosynthesized under restricted fermentation conditions where, the composition of the fermentation medium contains both very cheap constituents i.e., RP, FBS and $(NH_4)_2$ SO_4 andnutritionally poor and may cause environmental problem i.e., RS. The FMwas used here without any pretreatment; this is economically feasible and lead to cost reduction in the large-scale bioconversion. However, the major evidence for the bioconversion process was the hydrolysis of FM into soluble and insoluble sugars. Although, each organic acid has its own production pathway, some of them share the use of mono-sugars as the corner stone of bioconversion process. For instance, citrate and oxalate fermentations share similar physiology and each product is no more than one enzymatic step from the primary pathway of D-glucose and D-fructose metabolism, likewise, the mechanism leading to malate production is the same as the pathway leading to fumarate, abbreviated by one step, whereas, there are three possible metabolic mechanisms for production of succinate; the oxidative portion of the tricarboxylic acid cycle (TCA), the reductive portion of the TCA, or the glyoxylate bypass (El-Naggar and El-Hersh, 2011; Magnuson and Lasure, 2004; Saber *et al*., 2010). Formation of two major succinic and citric acids could be regarded as "semi homo-fermentation", since there are two types, hetero-fermentation of sugars to a mixture of end-produc (Rodrigues *et al*., 2013).

A. oryzae S2, strain with accession number KJ487973, was molecularly identified which is high sensitive and specific for identifying microorganisms and can be used for classifying microbial strains at diverse hierarchical taxonomic levels. These techniques offer a rapid tool for identification of filamentous fungi using two specific PCR primers sets. The sequence that is exemplified in the detailed description covers the 18S rRNA gene, through the internal transcribed spacer; each species has a unique nucleotide sequence through this stretch of genes (Turenne *et al*., 1999; Sette *et al*., 2006).

Residual biomass i.e., RS is poor in nutritional value and has complex composition which restrict its use by microorganisms in several biotechnological aspects, such as production of organic acids. Supplementation of such residual biomass with additional nutrients, may be cost effective, on the other hand, pretreatment may be cost effective and time consuming as well. Alternatively, co-inoculation by complimentary microorganisms, is an innovative technique, provides simple wayfor fermentation of residual biomass and production of organic acids (succinic and citric acids) by the synergism between the cellulose degrading *A. oryzae* and the N-fixing *Azotobacter chroococcum*. To the best of our knowledge, this is the first report in this respect and no pervious study dealt with such topic.

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