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Research Article
A Dual Microbial Culture for Improving C/N Ratio and Multi-response Optimization of Rice Straw Fermentation for Bioethanol Production

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Abstract
Background and Objective: Utilization of Rice Straw (RS) as an abundant and cheap source of biofuel production is a good example of the second generation of biofuel but its high C/N ratio is one of its disadvantages. This study was aimed to optimize this ratio via synergistic model between *Penicillium purpurogenum* MM70 and *Azotobacter chroococcum*. **Materials and Methods:** Solid state fermentation of RS by *P. purpurogenum* MM70 and *A. chroococcum* with the aid of Rock Phosphate (RP), $(NH_4)_2SO_4$ and Faba Bean Straw (FBS) was used according to Box-Behnken statistical design. The method has been adopted for multi-response surface optimization for biodegradation of RS using the desirability function approach. Design-Expert version 7 was used for both constructing the design and statistical analysis of experiments. **Results:** Solid state fermentation for nine days was optimal for improving C/N ratio, also for higher production of glucose and hydrolysis of biomass. The optimum experimental design was valid at levels of RP at 250 mg as P_2O_5, $(NH_4)_2SO_4$ at 50.0 mg and FBS at 0.90 g per 10 g RS. The obtained responses recorded 35.31±0.21% residual of Fermented Matter (FM), 40.37±1.03 μg^{-1} FPase and 8.57±0.35 mg of free sugars per g^{-1} FM., which successfully fermentedto12 g L^{-1} bioethanol by *Saccharomyces cerevisiae* **Conclusion:** Synergistic effect of *A. chroococcum* with the hydrolytic activity of *P. purogenium* MM70 is a simple and low-cost method in C/N ratio optimization of RS, production of cellulases and subsequently increase the amount of produced glucose, which can be used in bioethanol production.

Key words: Rice straw, C/N ratio, Box-Behnken design, bioethanol, *Penicillium purpurogenum* MM70

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Nowadays, the opposite relationship between the excess need of energy and the limitation of traditional sources (fossil fuel), derived the researchers to nonconventional and renewable sources of energy.

First-generation bioethanol associated with fermentation of sugar and starch sourced from crops such as sugar cane, wheat and corn, which contain a high concentration of sugar[1,2]. However, these crops are also important food sources, therefore the production of ethanol from these crops acting as significant impact on food prices and food security[2]. On the other hand, lignocellulosic wastes are produced in huge amounts worldwide and cellulose is the most abundant renewable polymer, which constitutes a large fraction of the lignocellulosic wastes[1]. In Egypt and all over the world, Rice Straw (RS) is the largest portion of available biomass feedstock contain a large amount of cellulose (around 33%)[3]. The RS as a lignocellulosic agro-residue (second generation) can be the cornerstone in bioethanol production by more research in bioconversion platform, which comprising of pre-treatment, enzyme production, saccharification and fermentation[4]. In bioethanol production, the most important and expensive factor (40%) is the availability of catalytically efficient mixture of glycosyl hydrolases (cellulases/hemicellulases) capable of releasing fermentable sugars from RS[5]. Therefore, bioprospecting for novel microbial strains capable of producing a variety of cellulases enzyme is an area of intensive research[6].

Many fungi produce very effective cellulases and the key factors such as temperature, aeration, moisture and nutrients as well as the carbon-nitrogen ratio (C/N ratio) are essential for fungal growth and cellulase production. However, one of the limiting factors in growing of microorganisms on RS and production of cellulases is the limitation of nitrogen source which can be expressed in high CN ratio (<65) where it should be at 25-30 as initial optimum ratio[2]. The classic way to solving this problem is the adding of external organic or inorganic source of nitrogen, which increased the cost of the process. Instead of adding external nitrogen sources, the present study aim to elevation of the nitrogen content and improve the C/N ratio of RS by co-fermentation by *P. purpurogenum* MM70 and *A. chroococcum* via Solid-State Fermentation (SSF) which have several advantages over submerged fermentation, such as higher productivity, higher product stability, lower catabolic repression and lower demand on sterility due to the low water activity in SSF[7-9]. The aim of study was to optimize the fermentation process using Box-Behnken design in this study. Ultimately, the ethanol production by *S. cerevisiae* has been investigated.

MATERIALS AND METHODS

Microorganisms, rock phosphate and rice straw: The certified and authentic strain of non-symbiotic nitrogen-fixing bacterium; *A. chroococcum*, checked for nitrogenase activity on the medium of Schmidt and Belser[10], was provided by Biofertilizers Unit, Department of Microbiology, Research Institute of Soils, Water and Environment, Agricultural Research Center, Giza, Egypt.

The cellulolytic fungus, *P. purpurogenum* MM70 was obtained from Dr. Mohammad M. El-Metwally, fungal collection at Mycology Laboratory, Faculty of Science, Damanhour University, Egypt.

Rock Phosphate (RP), containing 7.97% phosphorus (P), was obtained from Soils, Water and Environment Research Institute, Agricultural Research Center, Giza, Egypt. Rice Straw (RS) and Faba Bean Straw (FBS) were collected from Tag Elezz Agricultural Research Station, Dakahlia, Egypt.

Preparation of microbial inocula: The inoculum was prepared by growing *A. chroococcum* in modified Asby's medium[11] with shaking at 30°C for 48 h to obtain 10^8 mL^{-1}colony forming unit. Whereas *P. purpurogenum* MM70 was inoculated on potato dextrose agar plates and incubated at 30°C for 7 days, then 10 mL of sterile distilled water was added and the fungal colonies were scraped by the tip of a Pasteur pipette, the spore suspension was adjusted to about 10^7 spore mL^{-1}. The antagonism between *P. purpurogenum* MM70 and *A. chroococcum* was investigated to avoid the incompatibility issue.

Box-Behnken design and fermentation procedure: The fermentation medium contained 10 gm of RS as a fixed variable. The independent components were rock phosphate (RP, X_1), $(NH_4)_2SO_4$ (X_2) and Faba Bean Straw (FBS, X_3). No any additional constituents were added to the fermentation medium. The suitable fermentation time was first investigated using a medium containing 10 g of RS in addition to the middle concentrations of the independent variable, the fermentation lasted for 12 days. The carbon to nitrogen (C/N) ratio and liberated reducing sugars were used as the base for the determination of incubation period. Organic matter was determined according to the method described by Black[12]. Total nitrogen was determined by using the microkejeldahl method as described by Pregl[13].

A Box and Behnken[14] statistical design was adopted on three independent variables using the software Design-Expert version 7 (Stat-Ease, Minneapolis, USA). Each of the independent variable (RP, $(NH_4)_2SO_4$ and FBS) was studied at three coded levels i.e., -1, 0 and +1 for low, middle and high

Table 1: Box-Behnken matrix of the independent variables per 10 g RS showing the various responses as a result of dual inoculation by *P. purpurogenum* MM70 and *A. chroococcum*

	Tested independent variables			Response variables		
Run	RP (mg P_2O_5) (X_1)	$(NH_4)_2SO_4$ (mg) (X_2)	FBS (g) (X_3)	Residual FM (%)	FPase (Ug^{-1} FM)	Glucose (mg g^{-1} FM)
1	50 (-1)	10 (-1)	1.0 (0)	68.46	6.00	1.935
2	250 (1)	10 (-1)	1.0 (0)	54.62	19.38	4.902
3	50 (-1)	50 (1)	1.0 (0)	77.69	3.00	0.774
4	250 (1)	50 (1)	1.0 (0)	33.85	39.46	9.288
5	50 (-1)	30 (0)	0.5 (-1)	71.54	5.08	1.548
6	250 (1)	30 (0)	0.5 (-1)	50.00	27.69	5.418
7	50 (-1)	30 (0)	1.5 (1)	72.31	3.23	0.774
8	250 (1)	30 (0)	1.5 (1)	46.92	28.85	7.224
9	150 (0)	10 (-1)	0.5 (-1)	67.69	4.62	1.806
10	150 (0)	50 (1)	0.5 (-1)	56.92	19.27	4.644
11	150 (0)	10 (-1)	1.5 (1)	63.08	11.08	2.838
12	150 (0)	50 (1)	1.5 (1)	63.85	7.50	4.386
13*	150 (0)	30 (0)	1.0 (0)	63.08	17.42	3.096
14*	150 (0)	30 (0)	1.0 (0)	56.92	16.27	3.612
15*	150 (0)	30 (0)	1.0 (0)	59.23	18.23	3.354
16*	150 (0)	30 (0)	1.0 (0)	56.92	16.27	3.612
17*	150 (0)	30 (0)	1.0 (0)	59.23	18.23	3.354

*The middle points, RP: Rock phosphate, FBS: Faba bean straw, FM: fermented matter, FPase: Filter paperase

concentration, respectively. The coded and uncoded levels, as well as the design matrix of a 17 trials experiment, are presented in Table 1 and the second order polynomial quadratic model of the three independent factor is[14]:

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

where, Y is the predicted response, β_0 model constant; X_1, X_2 and X_3 are 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.

The fermentation medium was autoclaved at 121°C for 20 min and the inoculated with 5% (v/w) from each of both *P. purpurogenum* MM70 and *A. chroococcum*. The initial moisture content was adjusted to about 65%. The contents were mixed thoroughly and incubated at 30°C.

Based on the suitable bioconversion period obtained from the previous time course, the incubation of Box-Behnken experimental design was carried out for 9 days. After incubation, 100 mL of distilled water was added to each flask, shaken for 30 min. on a rotary shaker at 140 rpm and filtered through Whatman No.1 filter paper, then centrifuged at 5000 rpm for 10 min. The filter paperase activity and glucose were assayed in this filtrate and the residual percentage of the fermented biomass (FM) was determined.

Assay of filter paperase: Filter paperase activity (FPase) was assayed by incubating 1 mL of filtrate with 50 mg filter paper Whatman No. 1 (1.0×6.0 cm) in 1 mL of 0.2 mol acetate buffer (pH 4.8) at 50°C for 60 min. The reducing sugars released were estimated in the supernatant by 3,5-dinitrosalicylic acid assay[15]. One unit of FPase activity (U) correspond to 1 µ mol of glucose equivalent released per minute per gram of fermented biomass under the experimental assay conditions.

Desirability function for multi-response surface optimization: For simultaneous optimization of the tested responses of the three tested variables, 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. All the calculated values were experimentally validated in triplicate.

Bioethanol production: The bioconversion process of fermented biomass into bioethanol was performed using the optimum concentration of the independent variables, which were calculated and validated based on the overall desirability value for multi-response surface optimization. Considering the optimum conditions from the previous optimization trials, a bulk of the fermented sugary material was prepared, to be biologically converted into bioethanol production by the yeast strain *Saccharomyces cerevisiae* RTL543.

The tested sugary material was supplemented with nitrogen and phosphorus as follow; KH_2PO_4 0.1%, $(NH4)_2SO_4$ 0.5%, $MgSO_4.7H_2O$ 0.05% and yeast extract 0.1%. The pH of the medium was adjusted to 5.0 and the prepared sugary syrup was put in the fermenter followed by the yeast inoculum and incubated at 30°C. Air flow was provided through a port that entered through the top of the fermentor (for about 2 h) to allow the yeast to grow and reproduce. Samples were extracted from the fermentor every 1 h, therefore to measure the changes in temperature, pH and density of the solution. When two followed similar results were obtained the fermentation process was stopped. The fermentation broth was obtained in order to measure the ethanol and remaining sugar concentrations[16]. The fermentation efficiency of the obtained sugary material was estimated as follows:

$$\text{Fermentation efficiency} = \frac{\text{Actual ethanol recovery}}{\text{Theoretical recovery}} \times 100$$

Theoretical recovery = Total sugars × 0.64
Actual ethanol recovery = Actual ethanol obtained

Statistical analysis: The statistical software package Design-Expert version 7 (Stat-Ease, Minneapolis, USA) was used for both constructing the design and statistical analysis of experiments at probability (p) value <0.05, as well as for solving the multi-response surface problem to find out the optimum concentration of each independent variable.

RESULTS

Respecting to the antagonism between the fungus *P. purpurogenum* MM 70 and the bacterium *A. chroococcum*, the dual growth of the two organisms was conducted on PDA plates to investigate their ability to grow in the synergistically. Interestingly, no visible antagonism has been observed (data not shown). Wherein, the bioconversion trial using a dual inoculation has been designed during the subsequent solid-state fermentation technique.

Time course of biodegradation of fermented biomass: Data of Fig. 1 revealed that the biodegradation process of fermented biomass (RS, RP, $NH_4(SO_4)_2$ and FBS) by the dual microbes was associated with marked changes in the residual FM and C/N ratio, with slight variation in the pH of the obtained hydrolysate, along with the incubation intervals.

A great reduction in residual biomass after 9 and 12 days of fermentation, recording 45.0 and 40.5% from the original biomass weight, respectively. As well as, the C/N ratio reduced from 68.94% after 3 days of fermentation to 30.78% after 12 days; however, there was acontinual reduction in both FM weight. Generally, the 9 days of fermentation process was considered as the chosen period for the optimization process, in which the C/N ratio decreased up to 20.83 and this was may be favorite for fungal growth, as well as the liberated reducing sugars that were produced in a highest value.

Optimization of RS biodegradation: Under solid-state bioconversion process supported with the aid of a dual inoculation (*P. purpurogenum* MM70 and *A. chroococcum*) the Box-Behnken design was adopted to explain the interaction among the three independent variables (at three different concentrations),viz., RP, $(NH_4)_2SO_4$ and FBS on the production of FPase and biodegradation of RS which expressed in residual FM and glucose production (Table 1). Run number 4 was the highest, in which the experimental values of residual FM, FPase and glucose were 33.85%, 39.46 Ug^{-1} FM and 9.288 mg g^{-1} FM, respectively.

The one-way analysis of variance of the obtained data is presented in Table 2. The overall model terms of the various responses were significant; where the p-values were lower than 0.0001. Contrarily, the p-value for the lack of fit error did not reach the level of significance, in which the p-value for any of the tested parameters was $\geq$0.191. The coefficient of determination (R^2), the adjusted R^2 and predicted R^2 values were high enough to validate the efficacy of the tested parameters (RP, $(NH_4)_2SO_4$ and FBS) and their levels in the

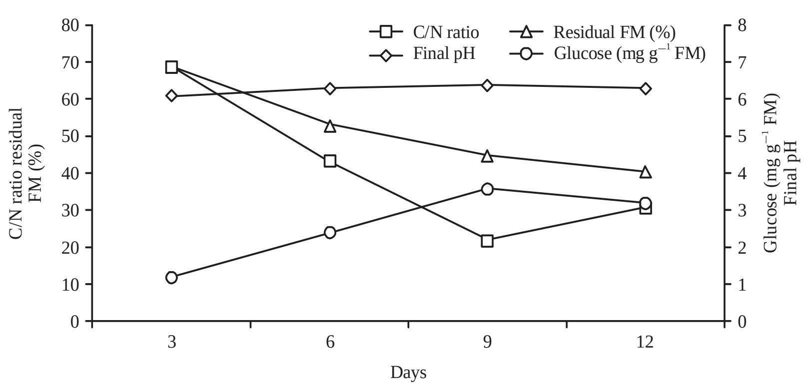

Fig. 1: Time course profile of biomass degradation of RS fermented with both *P. purpurogenum* MM70 and *A. chroococcum*

Table 2: Analysis of variance and coefficient of RS fermentation by *P. purpurogenum* MM70 and *A. chroococcum*, based on Box-Behnken matrix

	Residual FM (%)			FPase (U g^{-1} FM)			Glucose (mg g^{-1} FM)		
Source	Coefficient estimate	F-value	p<0.05	Coefficient estimate	F-value	p<0.05	Coefficient estimate	F-value	p<0.05
Model	81.37	31.50	<0.0001^{S}	-18.89	112.70	<0.0001^{S}	2.43	115.10	<0.0001^{S}
X_1	0.05	223.85	<0.0001^{S}	-0.06	740.87	<0.0001^{S}	-0.02	782.19	<0.0001^{S}
X_2	-0.03	9.49	0.0178^{S}	0.64	61.05	0.0001^{S}	-0.06	95.33	<0.0001^{S}
X_3	-27.15	0.00	1.0000^{NS}	39.60	2.77	0.1398^{NS}	1.37	5.37	0.0536^{NS}
X_1X_2	0.00	36.82	0.0005^{S}	0.00	82.03	<0.0001^{S}	0.00	101.28	<0.0001^{S}
X_1X_3	-0.02	0.61	0.4621^{NS}	0.02	1.39	0.2775^{NS}	0.01	21.91	0.0023^{S}
X_2X_3	0.29	5.45	0.0523^{NS}	-0.46	51.20	0.0002^{S}	-0.03	5.48	0.0518^{NS}
X_1^2	0.00	1.67	0.2371^{NS}	0.00	18.03	0.0038^{S}	0.00	18.06	0.0038^{S}
X_2^2	0.00	0.89	0.3777^{NS}	-0.01	22.73	0.0020^{S}	0.00	3.42	0.1069^{NS}
X_3^2	10.69	4.92	0.0620^{NS}	-14.84	35.70	0.0006^{S}	-0.94	3.07	0.1231^{NS}
Lack of fit		0.92	0.5079^{NS}		2.58	0.1912^{NS}		2.47	0.2014^{NS}
R^2		0.976			0.993			0.993	
Adjusted R^2		0.945			0.984			0.985	
Predicted R^2		0.820			0.924			0.927	
Adequate precision		21.70			36.90			41.12	
S.D		2.47			1.27			0.28	
C.V (%)		4.11			8.28			7.49	
PRESS		318.86			125.87			5.82	

S: Significant at p<0.05, NS: Not significant, S.D: Standard deviation, PRESS: Predicted residual sum of squares, FPase: Filter paperase

biodegradation process. The values of all kinds of R^2 are in reasonable agreement, recording $\geq$0.9, except the predicted R^2 value of the residual FM, being 0.820. However, the higher the R^2 the more accurate of the model design, which was confirmed by the high adequate precision values of the three responses. Finally, the Predicted Residual Sum of Squares (PRESS) values were relatively low, indicating the lower possibility of error during the experimental work.

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. The three independent variables (X_1, X_2 and X_3) were further statistically analyzed for interaction and cubic effect on the different response parameters. In this respect, the interaction of X_1X_2 was significant for all the tested response variables. However, the interaction of X_1X_3 was not significant for response variables, except that glucose production. The cubic X_1^2 was not significant for FM, but significant for the other responses. Additionally, the cubic X_2^2 was not significant for the response variables, except for FPase enzyme. As well as, the X_3 was not significant for the response variables. The other terms vary between significant and non-significant based on the nature of the independent variable.

Estimation of optimum level of each variable and validation: For solving the multi-response problem for calculation of optimum level for each of RP, $(NH_4)_2SO_4$ and FBS, the data were fitted using the second order polynomial equation. In practical cases, many situations encounter to multi-responses. In such cases surveying two or more response variables are critical, as in the presence case, which requires the optimization of the tested independent variables (X_1, X_2 and X_3) to yield the lowest residual FM and conversely optimization of FPase activity and the released glucose for the highest values, at the same time. The multiple response problems deal with such topic. Multiple response variables create difficulty because what is optimal for one response may not be optimal for the other responses.

The optimization was carried based on total desirability function D of the multi-response surface that lies between 0 and 1. 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. As the response approaches the target, the D value becomes closer to 1. To calculate D, the residual FM was set to be minimized and the FPase (Ug^{-1} FM) and glucose to be maximized. Solving this multi-response optimization problem led to obtaining the levels of RP at 250 mg as P_2O_5, $(NH_4)_2SO_4$ at 50.0 mg and FBS at 0.90 g per 10 g RS, as solid state. The theoretical values of the response variables were 35.06% residual FM, 39.46 Ug^{-1} FPase and 9.13 mg g^{-1} FM of free sugars, the corresponding d values were 0.972, 0.999 and 0.982, yielding a total D value of 0.984 (Fig. 2). The previously calculated data was experimentally validated; the obtained experimental responses recorded 35.31$\pm$0.21% residual FM, 40.37$\pm$1.03 Ug^{-1} FPase and 8.57$\pm$0.35 mg g^{-1} FM of free sugars.

Ethanol production during fermentation process by *S. cerevisiae*: Following the optimum levels at the desirability

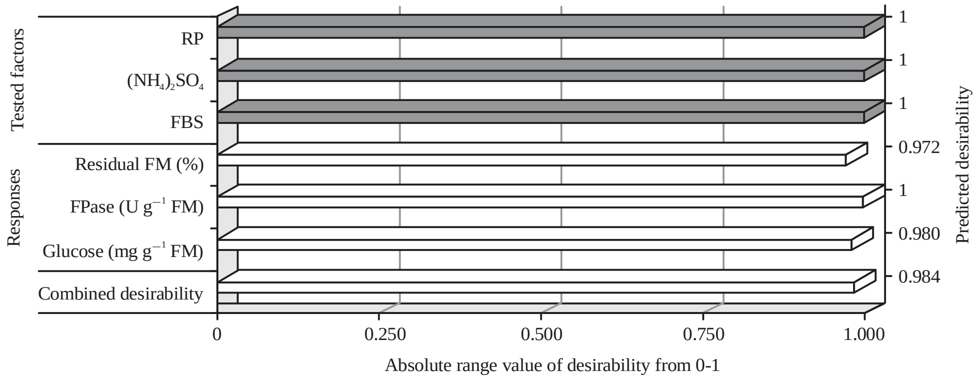

Fig. 2: Desirability for each tested factor and each response individually as well as the combined desirability of all the factors and responses

Table 3: Total reducing sugars of hydrolysate RS and ethanol content after fermentation by *S. servecieae*

Parameters	Values
Total reducing sugars (g L^{-1})	40.43
Theoretical recovery	25.875 = (40.43×0.64)
Ethanol content (g L^{-1})	12.0
Fermentation efficiency (%)	46.376

function (D), the fermented medium with the optimum concentrations of independent variables was constructed and inoculated by *S. cerevisiae*. After fermentation, the total reducing sugars, ethanol content and fermentation efficiency being 40.43,12 and 46.376 gL^{-1}, respectively (Table 3).

DISCUSSION

In this article, the substrate of fermentation is RS, which contain a considerable amount of cellulose (around 33%) but on the other hand high C/N ratio (>65 %) which is not suitable for good growth and cellulase production by the fungus. The two microorganisms, *A. chroococcum* and *P. purpurogenum* MM70 were selected based on the nitrogen fixing capability of the first one and the good productivity of CMCase, FPase and β-glucosidase for the second one[17]. The two organisms were grown in a compatible profile with no antagonistic trend and this synergistic growth reduced the C/N ratio to 20, which was suitable for microbial growth and biodegradation process[18]. This synergism is also more efficient than 35.2 C/N ratio carried by the addition of peptone and yeast in starch digesting *Aspergillus niger*[19].

The previous finding of Saber *et al.*[20], showed this trend of growth during the bioconversion of rice straw into organic acids using a dual inoculum of both *A. chroococcum* and *A. oryzae*. This synergism exploiting the efficacy of *A. chroococcum* in fixing the atmospheric nitrogen, which considered then the compensative source for both organic and inorganic source. Additionally, it is obvious from the data that, the 9 and 12 days were the fermentation intervals associated with a great reduction in FM% and C/N ratio.

The results also showed the efficacy of *P. purpurogenum* MM70 in the hydrolysis of fermented material, in which the FM% decreased up to 45 and 40% after 9 and 12 days, respectively, with the values of reducing sugars ranging between 3.6 to 3.2 mg g^{-1} FM. Wherein, the 9 days were chosen as the best interval period, which is coinciding with the finding of Rosyida *et al.*[21], as optimum incubation period in maximum production of cellulase during scarification of straw by *Trichoderma reesei*. Whereas, Mussatto *et al.*[22] found that the 4 days was optimum period for bioconversion of cellulosic substrate by the FPase enzyme. Further, the 6 days were the optimum period for bioconversion of sugarcane molasses into organic acids using *A. niger*[23].

Respecting to Box-Behnken design in the current study, the analysis of variance showed the model fitted using the second polynomial order, the overall model of the tested responses was significant. Similarly, R^2, predicted R^2 and adjusted R^2 were significant at $p<0.05$ for all tested responses. Whereas, the lack of fit error of p-value was not significant. All the previous values indicated that the model could be used efficiently to measure the particular model fits at each point in the design and validated the data of the different responses. Further, The SSF technique used in the Box-Behnken design has the advantage of reducing the cost of the medium, which depend upon using the lignocellulosic materials as a substrate, without needing the pure cellulose. The SSF, in comparison to liquid fermentation, allows the formation of a more highly concentrated product (in many cases in a shorter period) and has been exploited for the production of lignocellulolytic enzymes with agricultural wastes[24]. Moreover, SSF is providing simple growth conditions and control system with reduced energy consumption, which leads to a reduction in environmental impact and adds value to the by-products of agroindustry[8,9]. Commonly, the multi-response surface technique using desirability function showed the validity of the data and experiment to be applicable in different treatments of organic substances.

The cellulase productivity in our approach ranging between 3.00 to 39.46 FPase U g^{-1} on RS which is more higher than 7.2 Fpase Ug^{-1} recorded by *Aspergillus terreus*[25] and 19.5 FPase Ug^{-1} by *Aspergillus niger* KK2 mutant (KFCC 11285)[26].

Another interesting aspect of the current study is the impressive potential of *S. cerevisiae* to produce ethanol from saccharified rice straw by *P. purpurogenum* MM70. However, solid loading above 15% level may not result in greater cellulose conversion in SSF process, owing to high viscosity and mass transfer[27], the ethanol obtained during the fermentation process of cellulosic hydrolysate was 12 g L^{-1}. The efficiency of the *S. cerevisiae* in the conversion of biomass being 45.37%. Whereas, the reducing sugars content was 40.43 gL^{-1}. Surprisingly, the ethanol yield (12 g L^{-1}) was much higher when compared to 3.05 g L^{-1}, reported during saccharification and fermentation of sugarcane bagasse treated biologically with *Cerioporipsis* and *Phanerochaete*[28] and more than 8.85 g L^{-1} recorded by co-culture fermentation of tapioca flour as the substrate by *Aspergillus niger* and *S. cerevisiae*[19]. On the other hand, the ethanol yield was slightly lower than 16.5 g L^{-1} that reported by Mahajan *et al.*[29], who used *S. cerevisiae* in culture extract of *Malbranchea cinnamomea* to produce ethanol from alkali treated rice straw after supplementation of β-glucosidase from *Aspergillus* sp. ''O'' (35 cellobiase U g^{-1} substrate) and lower than 30.8 g L^{-1} resulted from enzymatic hydrolysis (The enzyme loading was set at 6.5 U g^{-1} FPase using a commercial cellulose) of RS and fermented by *Pichia stipites*[30]. The increase in bioethanol yield in the last two examples can be referred to use commercial cellulase enzymes, which make the processes more expensive.

CONCLUSION

Good management of microbial interactions can be directed in successful bioconversion of agro-waste. In this article both *A. chroococcum* and *P. purpurogenum* MM70 were grown in synergism mechanism, in which the C/N ratio decreased to a suitable value for growth and production of fungal cellulases which is the key factor in saccharification and subsequently production of bioethanol from RS. Additional work has to be done to use the nitrogen-fixing microbes in improving the C/N ration of different agro-wastes. This natural and inexpensive approach will make different agro-wastes suitable for microbial hydrolysis to many benefit compounds.

SIGNIFICANCE STATEMENT

This study revealed the impact of *A. chroococcum* in improving the C/N ratio of RS that can be beneficial in the hydrolysis of RS and other agro-wastes.

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