Application of Plackett Burman Design for Citric Acid Production from Pretreated and Untreated Wheat Straw

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ABSTRACT: *A solid state fermentation method was used to utilize wheat straw as substrates for citric acid production by using Aspergillus niger ATCC 9142. The Plackett Burman design (PBD) of experiments was used to test the relative importance of the variables affecting production such as moisture content, age of spore, inoculum size, initial pH of substrate, methanol concentration, incubation temperature and time, as well as initial sugar concentration, steam time and type of solvent. In best condition, the microorganism produced 47 g citric acid per kg dry crude wheat straw, with yield of 93 % based on the amount of fermentable sugar consumed. The effect of pretreatment of wheat straw with HCl, NaOH and urea on the yield of production was also investigated by using PBD. Acid, alkaline and urea pretreatment of wheat straw increased citric acid concentration to 115, 85.0 and 109.5 g/kg of dry wheat straw and yield to 97, 97 and 96 % (respectively) based on available sugar consumed. Finally, up scaling was achieved to a 20-L solid state fermentor (tray bioreactor) in which moisture was constant in gas phase and acid pretreated wheat straw was selected as the most efficient pretreated substrate. The produced acid concentration and yield in fermentor was 60.53 g/kg of dry wheat straw and 2.11 g/kg.day, respectively.*

KEY WORDS: *Solid state fermentation, Citric acid production, Pretreatment, Wheat straw, Plackett-Burman design.*

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INTRODUCTION

The use of lignocellulosic materials available in agricultural wastes as a source of raw material for citric acid production is of considerable interest because of their renewable nature and abundance [1]. In the last two decades, a considerable interest has been shown in using agricultural products and their wastes such as date syrup [2] and pulp [3], kiwifruit peel [4], apple and grape pomace [5-9], pineapple [10,11], mandarin orange [12], brewery wastes [13], corn cobs [14], coffee husk [15], kumara [16], okara [17], raw starch [18], sugarcane bagasse [19,11], semolina [20], and carob pod [21] for citric acid production by *Aspergillus niger*. Recently, *Kumar et al.* have reviewed the utilization of fruits waste for citric acid production by solid state fermentation (SSF) [11]. Wheat bran has been employed as a substrate in SSF and accounts for a fifth of the total citric acid production in Japan [22].

A number of carbon sources may be used for citric acid fermentation. For commercial reasons, the uses of molasses, sucrose or glucose syrups are favored. The use of molasses in particular is desirable because of its low cost availability [23].

Wheat straw is also a widely available substrate and its disposal entails an environmental problem, so transformation of this agricultural by-product is desirable. In many countries, including Iran, wheat straw is an abundant by-product from wheat production. Based on the data from FAO, 556.3 million metric tones of wheat were produced in the world in 2003. The average yield of wheat straw is 1.3-1.4 (% w/w) of wheat grain [24]. Wheat straw contains 30-45 % cellulose, 20-30 % hemicellulose and 6-15 % lignin [25]. Many researchers have been focused on the degradation of cellulose and lignin components from wheat straw and structural characterization of the hemicellulose fraction [26-29].

Citric acid, a tricarboxylic acid, is one of the world's largest tonnages of fermentation products [30]. It is widely used in the food and beverage industries as an acidifying and flavour-enhancing agent, pharmaceutical industry and elsewhere [9]. The entire worldwide demand for citric acid is met by fermentation mainly by the process involving the filamentous fungus *A. niger*. Citric acid is a commodity chemical, so, it is necessary to use inexpensive and readily available raw materials in industrial processes [31]. *A. niger* is the most commonly employed organism for citric acid fermentation [32].

SSF refers to the cultivation of microorganisms on solid materials in the absence of free liquid [33,34]. The major advantages of using SSF rather than submerged fermentation have been reported [33,34]. In citric acid production by *A. niger* in submerged fermentation, certain metal ions (Fe^{2+}, Mn^{2+}, Zn^{2+} etc.) are known to be inhibitory even at very low concentrations [35]. SSF gives high citric acid yield without inhibition related to presence of metals at high concentration [36]. *Shankaranand* and *Lonsane* even reported that addition of minerals into the production media to a certain level enhanced citric acid production. Therefore SSF is certainly a good way of utilizing nutrient rich solid waste as a substrate [15].

Acid pretreatment has become a state of the art technology for pretreating any lignocellulosic biomass [37]. It has the advantage of not only solublizing hemi-cellulose but also converting solublized hemi-cellulose to fermentable sugars [38]. However depending on the temperature, the acid pretreatment usually produces sugar degradation products, such as furfural, which are inhibitory to the fermentative microorganisms [39]. Compared to acid pretreatments, alkaline processes have less sugar degradation, furan derivative formation is avoided and many of the caustic salts can be recovered. Traditionally alkaline pretreatment increases the sugars yield obtained comparing untreated wheat straw [40].

For an efficient citric acid production, the growth of Aspergillus in pellet form is desirable and this can be achieved by process optimization [23]. A well defined statitistical experimental design is considered to be necessary for optimization of a fermentation process, since it would be possible to get more information through conducting fewer measurements during the process.

The *Plackett-Burman* design (PBD) has been frequently used for screening process variables that make the greatest impact on a process [41]. It is a set of small and efficient experimental design, which is very powerful, widely applicable and especially well suited for biotechnology research and development [42]. Recent reports on the use of PBD include its application toward improving antibiotic [43], *Saccharopolyspora spinosa* macrolide [44], *Colletotrichum coccodes* spores [45], succinic acid [37], xylanase [46], and

poly (hydroxybutyrtae) [47] as well as lipase catalyzed esterification [48]. However, best results could be obtained if optimization were focused on metabolic microorganism capacities [49]. A consideration in the choice of the PBD in screening studies is the ratio of the number of experiments to be conducted to the number of variables being studied. This design allows for the study of k=(N-1)/(L-1) factors, each with L levels with N experimental trials. The usefulness of the design lies in the fact that in determining the effects of one variable, the net effects of changing other variables cancel out so that the effect of each variable on the system can be independently determined.

The aim of this investigation was to compare the potential of crude with acid, alkaline and urea pretreated wheat straw as a source for citric acid production by *A. niger* via SSF as well as to study the the application of PBD to assess the relative importance of process variables such as moisture, temperature, methanol concentration, fermentation time, age of spore, initial pH and sugar, inoculum size, steam time and type of solvent.

EXPERIMENTAL

Organism and growth condition

A. niger ATCC 9142 was obtained from the culture collection, biotechnology group, the Iranian Research Organization for Science and Technology (IROST). The organism was maintained on potato dextrose agar (PDA) slants, preserved at 4 ºC, and subcultured every month.

Wheat straw was purchased from a local farmer and stored at room temperature until needed. The particle size of substrate was 0.5-3.5 cm. Then the components of the cell wall of the wheat straw were analyzed on the base of dried mass. It has been reported that the yield of citric acid production increased with increase of particle size, although larger particle size provides smaller surface area and allows good aeration [9]. Traditionally, the energy consumption for grinding to tiny particles is high [50]. Therefore, without cutting into small pieces, the substrate was ground and then dried in an oven. The wheat straw (4 g) was taken in 250 ml flasks, and supplemented with water and molasses to set the desired moisture and sugar level. Then media were autoclaved at 121 °C for 20 or 90 min to provide proper cooking of the substrate and to increase its susceptibility to microbial attack. Some colonies were inoculated on PDA slants and incubated at 30 ºC for 4 or 6 days. The spores obtained were suspended in 8 ml of sterile-distilled water to prepare the inoculum.

Pretreatments

For acid treatment wheat straw was slurried in 1 N or 5 N HCl, using a solid liquid ratio of 10 % (w/v), and pretreated in a water bath at 100 ºC for 1 h, after cooling down, the wheat straw was washed with distilled water and dried in an oven at 100 ºC.

Alkaline pretreatment of wheat straw was achieved by 1 M or 5 M NaOH solution, (solid-liquid ratio of 10 % w/v), during 1 h at room temperature. Then washed twice with distilled water, and dried in an oven at 100 ºC.

For urea pretreatment wheat straw was soaked with 2 % or 4 % (w/v) urea solution, using a solid-liquid ratio of 5 % (w/v), stored at room temperature for 3 weeks and dried in an oven at 100 ºC.

Solid-state fermentation

Experiments were conducted in 250-ml flasks, each containing 4 g of pretreated or crude wheat straw, and moistened with the appropriate amount of distilled water in order to contain 65 % or 75 % (v/w) moisture. Substrate was supplemented with sugar cane molasses to contain 14 % or 18 % (w/w) initial sugar (the water content of molasses was considered in moisture adjustment). Methanol was added at 3 or 4 % (v/w). The initial pH of the substrate was adjusted to 4 or 5.5 with 2 N NaOH or HCl (desired low and high value in table 1). After autoclaving at 121 ºC for 20 min or 90 min, the flasks were cooled to ambient temperature and inoculated with 1 ml of spore suspension containing about 10^5 or 10^7 spores/ml (low and high values in table 1). The flasks were incubated at 25 ºC or 30 ºC in an incubator for 4 and/or 5 days. Finally a predetermined concentration of nitrogen source (urea) was added to crude wheat straw in two levels (2 or 4 % w/w).

After screening the variables in flask for acid treated straw (with the highest efficiency of production), the experiment was scaled up in a 20 L solid state fermentor with constant moisture in gas phase. Fig. 1 shows the schematic diagram of scaling up and monitoring in tray bioreactor. The bioreactor was equipped to moisture and temperature recorder with a very long (up to 6 years long) memory to record any undesired changes in condition

Table 1: Variables to be monitored in citric acid production from wheat straw [(a)].

Variables	Low level (-)	High Level(+)
A: Urea concen.(% w/w)	2	4
B: Methanol (% v/w)	3	4
C: Steam time (min)	20	90
D: Initial sugar (% w/w)	14	18
E: Temperature (°C)	25	30
F: Time (day)	4	5
G: Solvent	water	acetone
H: pH	4	5.5
I: Inoculum size (spore/ml)	10^5	10^7
J: Moisture content (%)	65	75
K: Spore age (day)	4	6
In acid treatment[(a)] A: Acid concen. 10 % (v/w)	1 N	5 N
In urea treatment A: Urea concen. (% v/w)	2	4
In alkaline treatment A: NaOH concen. 10 %(v/w)	1M	5M

a) All the eleven factors except factor A are the same in untreated, alkaline, urea and acid treated wheat straw.

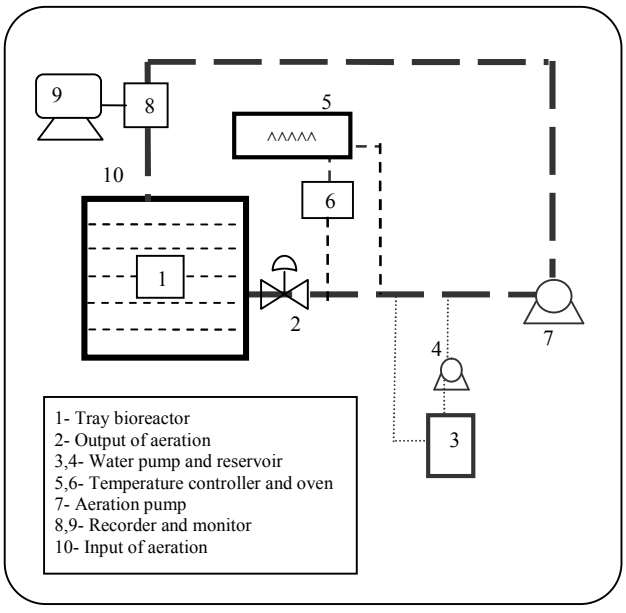

Fig. 1: Schematic diagram of experimental apparatus for solid state fermentation of sugarcane in a constant level of moisture in gas phase (not to scale).

which can occur due to default in controllers or disruption of electricity.

At the end of the fermentation time, the fermented materials were extracted with distilled water or acetone-distilled water solution (50 % v/v), in two stages on an agitator for 60 min. Then supernatant were separated via filtration, clarified by 10 min centrifugation at 3500 g and used for analysis of citric acid and residual sugar concentrations.

Analytical methods

The pH of the substrate before and after fermentation was measured by a pH-meter equipped with a glass electrode, using a solid-liquid ratio of 10 % (w/v) with distilled water. Moisture and ash content of the wheat straw were determined by drying the solid at 105 ºC and 550 ºC, respectively, to a constant weight [51]. The composition of wheat straw with respect to cellulose, hemicellulose and lignin content was determined with fibertech system [52] by the Animal Husbandry Research Institute of I.R. Iran. Crude protein of substrate was determined by micro-kjeldahl method [53]. Citric acid was determined photometrically at 420 nm by the acetic anhydride-pyridine method [54]. Total reducing sugars were measured by dinitrosalicylic acid (DNS) method, using glucose as standard [55]. Each experiment was repeated two times and the results were reported as averages of two repetitions.

Plackett-Burman Design (PBD)

The first screening step was to identify the variables which have significant effects on citric acid production by *A. niger*. In order to maximize *A. niger* growth and citric acid production, the effective factors and their levels were selected based on literature review and also our previous experience [8]. The important criteria to choose each factor settings for any two-levels screening design have been mentioned elsewhere [56,47].

The selected variables, which have shown in table 1 include some medium compositions (i.e., moisture content, initial concentration of carbon and nitrogen sources) and environmental factors (i.e., temperature, pH, fermentation time, inoculum size and age, solvent and time of steam treating). Table 2 shows selected experimental variables and a PBD for conducting 12 experimental trials. The elements, + (high level) and - (low level) represent the two different levels of the

Table 2: Twelve-trial PBD to study eleven factors in citric acid production from wheat straw [(a)].

Run No.	Codded setting for factors										
	A [(b)]	B	C	D	E	F	G	H	I	K	J
1	+	-	+	-	-	-	+	+	+	-	+
2	+	+	-	+	-	-	-	+	+	+	-
3	-	+	+	-	+	-	-	-	+	+	+
4	+	-	+	+	-	+	-	-	-	+	+
5	+	+	-	+	+	-	+	-	-	-	+
6	+	+	+	-	+	+	-	+	-	-	-
7	-	+	+	+	-	+	+	-	+	-	-
8	-	-	+	+	+	-	+	+	-	+	-
9	-	-	-	+	+	+	-	+	+	-	+
10	+	-	-	-	+	+	+	-	+	+	-
11	-	+	-	-	-	+	+	+	-	+	+
12	-	-	-	-	-	-	-	-	-	-	-

a) Factors A through K refer to those in table 1.
b) Four different tables of PBD has been constructed with four different factor A of Table 1 (acid, urea and alkali concen.) to compare untreated and treated straw.

independent variables examined. The PBD was constructed using a "generating vector" (+-+ + + --- + --) [57].

Range finding

One important factor that affects the performance of SSF is the moisture content of solids [21]. Decreasing the moisture level less than 65 %, may result a suboptimal product formation due to reduced mass transfer processes such as diffusion of solutes and gas to the cell during fermentation [58]. Although there is a lower limit of moisture content below which *A. niger* may not produce citric acid, due to higher osmotic pressure levels at lower moisture content [59]. The increase of moisture enhances the chance of contamination and decreases gas transformation due to the reduced inter-particle space [10]. To determine the effect of moisture on response two levels of 65 and 75 % were selected.

Another important factor is the initial pH of the substrate. The lowest value of pH was accompanied with the greatest concentration of citric acid, and the pH value increased due to oxidation of citric acid by the fungus [60, 61]. So in this research the range of initial pH was selected to be 4 and 5.5 to determine the effect of pH on response.

The addition of methanol at concentrations of 1-4 % (v/w) resulted in a marked increase in the amount of citric acid formed by *A. niger* on spent grain liquor and brewery wastes, respectively [62, 63]. The influence of methanol in increasing citric acid production appears to be a general phenomenon with dehydrogenase in an attempt to maintain an adequate intracellular level of the metabolite strains of *A. niger*, and the use of methanol has become a common practice [35]. The effect of methanol is at the cell permeability level, allowing citrate to be excreted from the cell; the cell then responds by increasing its citrate production via repression of 2-oxoglutarate dehydrogenase in an attempt to maintain an adequate intracellular level of the metabolite [64]. To determine the effect of methanol concentration on yield and productivity two levels of 3 and 4 % (v/w) was selected. Temperature and time have profound influences on the fungal production of citric acid from wheat straw. Two temperature levels of 25 and 30 ºC and duration of 4 and 5 days were selected to determine the relative importance of temperature and time, respectively.

Previous studies reported that initial sugar concentration of 14-22 % was optimum in industrial fermentations [65]. The available sugar concentration in wheat straw was about 4 %, which is much lower than the value of 14-22 %, so sugarcane molasses was added to increase the available initial sugar concentration to 14 % or 18 % (w/w). The initial sugar requirement for growth varies from species to species and strain to strain. The importance of this variable on citric acid production and growth has been reported [7,8]. Therefore, citric acid production was evaluated with different initial concentration of sugar.

Leaching is an important unit operation that extracts one or more constituents of a solid mixture by contact with a solvent and the countercurrent multiple contact system is commonly used to obtain the most concentrated solution of a product [66]. *Hang* and *Woodams* reported that acetone needed only 3 contact stages to leach the greatest amount of citric acid in SSF and gave an extraction efficiency of 90 %, moreover, acetone can be easily recovered and reused to reduce the cost of operation [66].

It has been established that the accumulation of citric acid requires a limitation in nitrogen source [67]. *Khare et al.* reported that the organic nitrogen sources such as peptone did not produce any significant increase in citric acid yield, however, the inorganic sources such as urea was presented the most effective resulting in 22.7 % more citric acid production than the control [17]. To determine the effect of addition of nitrogen source on yield and productivity, two levels of 2 % and 4 % (w/w) were selected.

Moreover, it is known that preparation and pretreatment are the requested steps to convert the agro-industrial residues into a suitable form to increase its utilisation by the microorganism. These include size reduction through grinding, rasping or chopping, physical, chemical or enzymatic hydrolysis of polymers to increase substrate availability, cooking or vapour treatment for macromolecular structure pre-degradation, and elimination of major contaminants [68].

Another variable which can affect the citric acid yield and productivity is condition of seed culture. The suitable condition for induction of ligninolytic activity can be achieved by selection of proper inoculum size and age. *Fernandez et al.*, in 1996 reported that a sharp decrease in citric acid production was observed with spores older than 7 days and that younger spores produced more biomass and citric acid than older ones [69]. *Fatemi and Shojaosadati* reported that increase of inoculum size, enhances the citric acid production, but further increase of inoculum size, decreases citric acid production due to consumption of substrate by more inoculated cells [7]. In this work the range of inoculum size and age were selected in 4 and 6 days and 10^5 and 10^7 (spore/ml), respectively.

For maximum citric acid production, crude and pretreated wheat straw were autoclaved for 20 and 90 min, before inoculation to increased hydrolysis of lignocellulose complex. It should be mentioned that addition of urea, NaOH and HCl does not have any effect on moisture because wheat straw was dried after treatment. Also in urea supplementation the moisture of urea solution was calculated to inhibit any noise in explanation of each separated effect.

RESULTS AND DISCUSSION

Wheat straw used in this investigation contained 33.8 % cellulose, 25.9 % hemicellulose, 6 % lignin, 12 % ash and 3.9 % crude protein on dry solid basis. By urea, alkaline and acid pretreatment of straw above mentioned ingredients were respectively changed as follows: 40.2, 52.7 and 58.7 % cellulose, 25.6, 18.6, and 15.2 % hemicellulose, 7.2, 8.4 and 8.4 % lignin. During fermentation period the sugar content of the medium decreased with the increase in the citric acid production. Biomass concentration increased rapidly in the first phase (4th day) and then a slight increase was observed. This was due to the exhaustion of nitrogen content [67]. The basic equation set up for the design was as follows. The coefficients for the eleven variables were determined by:

$$A_i = (1/N)\sum_{0}^{N} X_i \cdot K_i \quad (1)$$

where A_i = coefficient values, X_i = experimental yield, K_i = coded value of each variable corresponding to the respective experimental yield of X_i and N = number of experiments. Tables 3, 5, 7 and 9 give a comparison of the experimentally determined citric acid production yield and productivity to those predicted by solving the above equation, where the predicted yield is given by:

$$Y_i = \sum_{i=1}^{N} A_i \cdot K_i \quad (2)$$

for i = 0, a dummy level of +1 was used and the coefficient obtained was called A_0. The standard error was determined as the sum of the squares of the difference between the experimental and predicted yield for each run. The estimated error is given by:

$$S_b = \sqrt{S_e^2 / N} \quad (3)$$

The student's *t*-test was performed to determine the significance of each variable employed (*t*-value = coefficient/S_b). Since the experiments were designed to evaluate the relative effect of each variable on response, a significant level of 0.30 is acceptable [70]. However, the tabulated *t*-value (degree of freedom 10) at $P<0.1$ and $P<0.15$ is equal to 1.2 and 0.69, respectively. Statistical calculations for PBD of citric acid production from untreated wheat straw is summarized in table 4. Results show that sugar content of the medium was reduced by *A. niger* during fermentation and the amount of citric acid production increased in proportion with the sugar utilization. The amount of citric acid production and

Table 3: Comparison of experimented and predicted yield and productivity in untreated wheat straw [(a)].

Randomized	Citric acid yield % (acid/sugar)		Citric acid productivity (g/kg.day)	
	experimented	predicted	experimented	predicted
8	0.853	0.891	11.21	11.57
3	0.135	0.177	3.24	3.64
6	0.527	0.567	6.34	6.72
9	0.042	0.083	1.09	1.48
10	0.473	0.513	5.59	5.98
5	0.095	0.137	2.54	2.94
1	0.344	0.385	4.41	4.82
12	0.930	0.483	3.71	0.68
11	0.423	0.459	4.09	4.48
4	0.470	0.511	4.09	4.48
7	0.876	0.913	8.03	8.42
2	0.696	0.735	3.71	4.10

a) Each value is the average of two replications.

Table 4: Statistical data for analysis of variance of citric acid production from untreated wheat straw.

Variables \ Response	Citric acid yield [(a)] coefficient	t-value	Citric acid productivity [(b)] coefficient	t-value
A: Nitrogen source	-0.013	-0.097	0.01	0.01
B: Methanol (% v/w)	0.010	0.074	0.22	0.21
C: Steam time (min)	0.086	0.641	1.78	1.87
D: Initial sugar (% w/w)	0.057	0.425	0.67	0.71
E: Temperature (°C)	-0.093	-0.694	0.56	0.59
F: Time (day)	0.020	0.149	0.43	0.45
G: Solvent	0.062	0.462	1.54	1.62
H: pH	0.032	0.238	0.70	0.74
I: Inoculum size (spore/ml)	-0.020	-0.149	-0.09	-0.09
J: Moisture content (%)	-0.196	-1.462	-1.19	-1.25
K: Spore age (day)	0.060	0.447	0.88	0.93

a) A_0 = 0.488 (mean of the experimental yield), standard error, S_b = 0.134, estimated error, S_e^2 = 0.217, tabulated t-value (degree of freedom 10) at P< 0.1 and P< 0.15 is equal to 1.2 and 0.6, respectively. b) A_0 = 4.83 (mean of the experimental yield), standard error, S_b = 0.95, estimated error, S_e^2 = 10.85, tabulated t-value (degree of freedom 10) at P< 0.1 and P< 0.05 is equal to 1.3 and 1.8, respectively.

sugar consumption increased with an increase in methanol concentration. The highest amount of citric acid was produced in the presence of 3.0 % (v/w) of methanol in the fermentation medium. Citric acid concentration, sugar consumption, biomass concentration and citric acid yield increased in the presence of methanol. As table 4 shows the higher amount of methanol has increased the yield and productivity in citric acid production. The mould produced the greatest amount of citric acid from crude wheat straw in the presence of methanol at a concentration of 4 % (v/w). The influence of methanol in increasing citric acid production appears to be due to increased cell permeability and decreased end product repression of related enzyme. The results show that methanol concentration has a great impact on yield of citric acid production compared to its productivity. *Roukas* and *Kotzekidou* [63] reported that the addition of methanol at a concentration of 1.0 to 4.0 % resulted in increase of the amount of citric acid production by *A. niger* in spent grain liquor and brewery wastes. In another research, it was also reported that addition of 3.0 to 4.0 % (v/w) of methanol concentration retarded growth, delayed sporulation and increased citric acid yields [20].

The results of table 4 shows increased citric acid concentration followed by increasing the initial pH. Citric acid yield ($Y_{P/S}$) based on the sugar that was consumed at the end of fermentation were calculated (tables 3, 5, 7 and 9). The yield was defined by the amount of citric acid produced divided by the amount of sugar consumed. Also productivity was defined by the amount of citric acid produced divided by the amount of substrate consumed per day [20]. The microorganism produced 47.6 g citric acid per kg dry crude wheat straw, with yield of 93 % based on the amount of fermentable sugar consumed.

Table 4 shows that higher values of responses (yield and productivity) were achieved at a moisture level of 65 %. Lower moisture content reduces mass transfer to the cell and increases osmotic pressure, but increases inter-particle space stimulate citric acid production and decreases end product inhibitory. Also the initial pH of 5.5 for crude wheat straw results in higher citric acid yield and productivity than 4. The lower value of pH was accompanied with the higher concentration of citric acid, and increased pH value due to oxidation of citric acid by the fungus. Table 4 indicates that influence of lower pH on productivity was more significant than yield, which is due to stimulatory effect on fungus growth and citric acid production in higher pH.

Table 5: Comparison of experimented and predicted yields and productivities in acid pretreated wheat straw [(a)].

Randomized	Citric acid yield % (acid/sugar)		Citric acid productivity (g/kg.day)	
	experimented	predicted	experimented	predicted
8	0.770	0.836	11.45	12.45
3	0.693	0.760	11.91	12.89
6	0.862	0.928	7.28	8.29
9	0.616	0.684	11.59	12.59
10	0.973	1.040	15.15	16.09
5	0.922	0.990	27.38	28.35
1	0.919	0.984	19.65	20.63
12	1.000	0.262	8.40	2.59
11	0.928	0.994	11.78	12.79
4	0.764	0.832	5.97	7.01
7	0.698	0.766	7.28	8.27
2	0.587	0.656	6.29	7.29

a) Each value is the average of two replications.

Table 6: Statistical data for analysis variance of citric acid yield and productivity from acid pretreated wheat straw.

Responses / Variables	Citric acid yield [(a)] coefficient	t-value	Citric acid productivity [(b)] coefficient	t-value
A: Acid concen.	0.094	0.423	2.61	1.35
B: Methanol (% v/w)	0.038	0.171	0.97	0.50
C: Steam time (min)	0.040	0.180	-0.42	-0.21
D: Initial sugar (% w/w)	-0.017	-0.076	0.65	0.33
E: Temperature (°C)	0.062	0.279	3.11	1.62
F: Time (day)	0.063	0.283	-1.16	-0.60
G: Solvent	0.124	0.558	4.43	2.31
H: pH	0.036	0.162	0.33	0.17
I: Size of inoculum	0.004	0.018	0.96	0.50
J: Moisture content (%)	0.063	0.283	3.70	1.92
K: Spore age (day)	0.042	0.189	-0.58	-0.30

a) A_0 = 0.811 (mean of the experimental yield), standard error, S_b = 0.222, estimated error, S_e^2 = 0.594, tabulated t-value (degree of freedom 10) at P< 0.1 and P< 0.15 is equal to 1.2 and 0.6, respectively.
b) A_0 = 12.01 (mean of the experimental yield), standard error, S_b 1.92, estimated error, S_e^2 = 44.60, tabulated t-value (degree of freedom 10) at P< 0.1 and P< 0.05 is equal to 1.3 and 1.8, respectively.

Temperature has a considerable effect on the fungal production of citric acid on the wheat straw. The suitable fermentation temperature and time for citric acid production by *A. niger* ATCC 9142 grown on crude wheat straw were found to be 25 ºC and 5 days, respectively. These results agree with reports showing that the temperature did not strongly affect the growth rate in SSF in the range of 28-34 ºC [71, 72].

There was no significant difference between fermentation time of 4 and 5 days and temperature of 25 and 30 °C. Although increased temperature and time caused a slow increase in citric acid production in bench scale, but they may result in a time and energy consuming process in industrial scale [72]. Table 4 Also indicates that initial sugar concentration of 18 % (w/w) produced more citric acid than 14 % (w/w). This table also shows that inoculation of 6 days old spore and a spore suspension of about 10^5 (spores/ml) results increased yield and productivity of citric acid production.

Tables 5, 7 and 9 give a comparison of the experimented and predicted yields and productivities in acid, urea and alkaline pretreated wheat straw. Citric acid production increased to 115 g per kg of acid-pretreated wheat straw. The maximum yield of citric acid production which was related to trial no 5 was 97 % based on the amount of fermentable sugar consumed and the maximum productivity from trial no 6 also, increased to about 27.38 (g/kg.day).

Tables 6, 8 and 10 show statistical data for analysis of variance of citric acid production from acid, urea and alkaline pretreated wheat straw, respectively. In the 2nd columns of these tables some of the variables exhibited *t*-values lower than 1.2 implying that variables in the selected range were not significant on product yield. Column b of the tables shows the relative effect of each variable on citric acid productivity. Acid pretreatment increased citric acid concentration to 115 g/kg of dry wheat straw and citric acid yield to 97 % based on sugar consumed. The suitable level of variables including initial sugar concentration, pH, methanol concentration, incubation time, temperature, and solvent was the same for productivity of acid production from untreated and pretreated wheat straw. The effects of other variables show different impact depending on their levels, i.e. the higher productivity was observed in moisture content of 65 and 75 %; steam time, 20 and 90 min; inoculum size,

Table 7: Comparison of experimented and predicted yields and productivities in urea pretreated wheat straw [(a)].

Randomized	Citric acid yield % (acid/sugar)		Citric acid productivity (g/kg.day)	
	experimented	predicted	experimented	predicted
8	0.87	0.90	17.54	18.41
3	0.62	0.65	13.79	14.67
6	0.39	0.42	5.59	6.49
9	0.36	0.39	9.53	10.45
10	0.75	0.78	6.53	7.43
5	0.96	0.99	25.50	26.37
1	0.34	0.38	7.58	8.47
12	0.91	0.39	6.05	3.73
11	0.41	0.44	9.72	10.59
4	0.26	0.29	6.16	7.03
7	0.66	0.69	11.90	12.79
2	0.29	0.33	8.40	9.31

a) Each value is the average of two replications.

Table 8: Statistical data for analysis variance of citric acid yield and productivity from urea pretreated wheat straw.

Responses / Variables	Citric acid yield [(a)]		Citric acid productivity [(b)]	
	coefficient	t-value	coefficient	t-value
A: Urea concen.	-0.023	-0.151	0.160	0.148
B: Methanol (% v/w)	0.033	0.217	2.683	2.481
C: Steam time (min)	0.001	0.010	0.626	0.579
D: Initial sugar (% w/w)	0.045	0.296	3.371	3.118
E: Temperature (°C)	0.135	0.888	3.280	3.034
F: Time (day)	-0.050	-0.328	-1.561	-1.444
G: Solvent	0.143	0.940	3.328	3.078
H: pH	-0.078	-0.513	-0.073	-0.067
I: Size of inoculum	-0.018	-0.118	-0.178	-0.164
J: Moisture content (%)	-0.030	-0.197	2.246	2.077
K: Spore age (day)	0.011	0.072	0.556	0.514

a) A_0 = 0.560 (mean of the experimental yield), standard error, S_b = 0.152, estimated error, S_e^2 = 0.280, tabulated t-value (degree of freedom 10) at P< 0.1 and P< 0.15 is equal to 1.2 and 0.6, respectively. b) A_0= 10.69 (mean of the experimental yield), standard error, S_b = 1.081, estimated error, S_e^2 = 14.02, tabulated t-value (degree of freedom 10) at P< 0.1 and P< 0.05 is equal to 1.3 and 1.8, respectively.

Table 9: Comparison of experimented and predicted yields and productivities in alkaline pretreated wheat straw [(a)].

Randomized	Citric acid yield % (acid/sugar)		Citric acid productivity (g/kg.day)	
	experimented	predicted	experimented	predicted
8	0.816	0.871	10.98	11.73
3	0.133	0.188	3.01	3.80
6	0.979	1.032	7.28	8.04
9	0.879	0.931	10.65	11.42
10	0.818	0.873	11.03	11.80
5	0.883	0.938	19.88	20.64
1	0.682	0.735	10.51	11.30
12	0.208	0.396	5.11	3.52
11	0.621	0.674	5.59	6.38
4	0.900	0.953	14.59	15.34
7	0.826	0.880	8.22	9.00
2	0.192	0.248	6.29	7.10

a) Each value is the average of two replications.

Table 10: Statistical data for analysis variance of citric acid yield and productivity from alkaline pretreated wheat straw.

Responses / Variables	Citric acid yield [(a)]		Citric acid productivity [(b)]	
	coefficient	t-value	coefficient	t-value
A:NaOH concen.	0.136	0.751	2.953	3.381
B: Methanol (% v/w)	-0.0006	-0.003	-0.265	-0.303
C: Steam time (min)	0.116	0.640	0.455	0.521
D: Initial sugar (% w/w)	0.143	0.790	3.125	3.583
E: Temperature (°C)	0.145	0.801	1.828	2.096
F: Time (day)	0.230	1.270	0.916	1.050
G: Solvent	0.168	0.928	2.391	2.741
H: pH	0.088	0.486	-0.090	-0.103
I: Size of inoculum	-0.018	-0.099	-0.358	-0.410
J: Moisture content (%)	0.076	0.419	2.061	2.363
K: Spore age (day)	-0.026	-0.143	-0.061	-0.069

a) A_0 = 0.661 (mean of the experimental yield), standard error, S_b = 0.181, estimated error, S_e^2 = 0.395, tabulated t-value (degree of freedom 10) at P< 0.1 and P< 0.15 is equal to 1.2 and 0.6, respectively. b) A_0 = 9.428 (mean of the experimental yield), standard error, S_b = 0.875, estimated error, S_e^2 = 9.130, tabulated t-value (degree of freedom 10) at P< 0.1 and P< 0.05 is equal to 1.3 and 1.8, respectively.

10^5 and10^7 spores/ml; age of spore, 6 and 4 days from untreated and treated straw, respectively.

Result of tables 4, 6, 8 and 10 indicate that, acetone is more effective than water for leaching of the citric acid from the crude and pretreated wheat straw. This result verified *Hang* and *Woodams* report that indicated acetone needed only 3 contact stages to leach the greatest amount of citric acid in SSF and gave an extraction efficiency of 90 % [66]. These tables show that more steam time (contact of straw with steam of 121 °C and 15 psi) increased yield and productivity, which may be due to increased thermal hydrolysis of lignocellulose complex and enhanced bioavailability of sugars in substrate.

Conditions for suitable citric acid production using NaOH (5 M) pretreated wheat straw includes: sugar concentration of 18 % (w/w); pH 5.5; moisture level 75 %; methanol concentration: 3 % (v/w); age of spore 4 days; inoculum size 1×10^5 (spores/ml); incubation time 5 days and incubation temperature 30 ºC. Under these conditions, 85 g citric acid was produced from 1 kg dry wheat straw with yield of 97 % based on the amount of fermentable sugar consumed (trial no 3) and productivity of about 20.5 g/kg.day (trial no 6). The comparison of different applied strategies for pretreatment of wheat straw is shown in Fig. 2. Which indicates that different pretreatment methods cause significant increase in citric acid production in terms of yield (acid production based on the amount of straw consumed) and productivity. As figure shows there is no significant difference in the yield of acid production based on the amount of fermentable sugar consumed.

The maximal citric acid concentration, 109.5 g from 1 kg urea (2 % w/w) pretreated wheat straw, citric acid yield of 96 % based on consumed sugar (trial no 5) and productivity of about 25.5 g/kg.day (trial no 5) were obtained at a moisture level of 65 %, pH 4, at 30 ºC and initial sugar concentration of 18 % (w/w) after incubation time of 4 days, using 6 day-old spore suspension of 1×10^5 spores/ml in the presence of 4 % (v/w) methanol.

Finally, up scaling was achieved to a 20-L solid state fermentor (tray bioreactor) in which moisture was constant in gas phase (75 %) and acid pretreated wheat straw was selected as the most efficient pretreated substrate. All variables and their levels were set in their suitable conditions obtained from the results of acid pretreatment in flasks. The produced acid concentration and yield in fermentor was 60.53 g/kg of dry wheat straw and 2.11 g/kg.day, respectively.

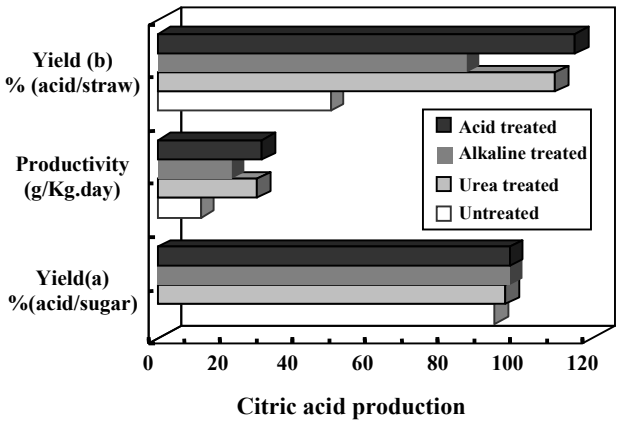

Fig. 2: Comparison of citric acid production in different applied strategies for pretreatment of wheat straw. a) yield of acid production based on the amount of fermentable sugar consumed; b) yield of acid production based on the amount of straw consumed.

CONCLUSIONS

In this research some important aspects of citric acid production from wheat straw by *A. niger* in SSF was evaluated. It can be concluded that acid pretreatment plays the most important role in the optimization of citric acid production from wheat straw. The concentration of produced citric acid was increased using 5N HCl-treated wheat straw. The suitable conditions for HCl-pretreated wheat straw fermentation were: initial sugar concentration 14 % (w/w); initial pH 5.5; moisture content 75 %; methanol concentration 4 % (v/w); steam time 90 min; inoculum size 10^7 (spores/ml); age of spore 6 days; incubation time 5 days; incubation temperature 30 ºC and leaching solvent was acetone.

A. niger ATCC 9142 produced 47.6 g citric acid per kg of dry crude wheat straw fermented. The maximum yield of citric acid production was 93 % based on the amount of fermentable sugar consumed, and the maximum productivity was about 12 (g/kg.day).

This process yielded 115 g citric acid/kg dry acid pretreated wheat straw. Based on the amount of sugar consumed, yield of 97 % and production of 27.38 (g/kg.day) were obtained. The spent residue left after the leaching of the citric acid can be decontaminated to kill the microorganism and then used as an animal feed component; the SSF may reduce the concentrations of

anti-nutritional factors [15]. These results suggest that the use of wheat straw for fungal production of citric acid could represent an efficient method for minimizing the wheat straw disposal problems and concomitantly producing a commercially valuable organic acid.

Among the variables, the steam time, moisture content and solvent were found to be the most significant variables affecting citric acid production productivity from crude straw (table 4). Also solvent, moisture content, temperature and acid concentration were the most significant variables affecting productivity from acid treated wheat straw (table 6). The most significant variables affecting productivity from urea pretreatment were solvent, moisture content, incubation time, temperature, initial sugar and methanol concentration. For alkaline treated wheat straw, solvent, moisture content, incubation time, temperature, initial sugar and alkaline concentration significantly affected the responses. However any response to each variable depends on the selected range. In fact in PBD or other screening designs, dummy or null variables may occur if the difference between two levels of each variable is not large enough to ensure a measurable response. Some sensitive variables on the other hand may have their high and low levels chosen such that the size of their differential response is so great as to mask the effect of other variables. Since the PBD is typically used as a preliminary optimization technique, more accurate quantitative analysis of the effect of these variables for citric acid production is required. Further studies have been planned based on the use of different strategies for pretreatment and central composite design to study only screened effective variable of moisture content and its supplementation with other nitrogen sources to increase citric acid yield. Citric acid production in solid state fermentor was still lower than in flasks, showing that complementary studies should be conducted.

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