Alfarama Journal of Basic & Applied Sciences

Faculty of Science Port Said University

https://ajbas.journals.ekb.eg
ajbas@sci.psu.edu.eg
http://sci.psu.edu.eg/en/

January 2023, Volume 4 , Issue I

DOI:10.21608/ajbas.2022.140194.1101

ISSN 2682-275X

Submitted: 23-05-2022
Accepted: 22-06-2022

Pages: 110- 120

Solid-state Hydrolysis and Fermentation of Rice Straw and Manure for Bioethanol Production

Kholoud M. El-Adrosy [1,*], Mohsen E. Ibrahim [1], Omar A. Abdul Wahid[2], Ahmad D. El-Bassuony[2]

[1] Department of Botany, Faculty of Science, Port Said University, Port Said, 42524, Egypt.

[2] Department of Botany, Faculty of Science, University of Suez Canal, Ismailia, Egypt.

***Corresponding author:** Kh_eladrosy@sci.psu.edu.eg

ABSTRACT

Bioethanol production from lignocellulose biomass is a suitable alternative to replace fossil fuels. In Egypt, rice straw and manure are the large resources of lignocellulosic biomass from agriculture and animal wastes. This study aimed to improve the bioethanol production from rice straw and manure under solid-state conditions, using the co-culture of *Streptomyces aegyptia* and *Candida tropicalis*. Improving the conversion process is controlled by a large number of factors such as medium volume optimization and strain improvement. The response surface methodology (RSM) was applied to determine the optimal medium volume in hydrolysis and fermentation processes for maximum ethanol production. The maximum ethanol concentration of 4.4 g/l (22 mg/g) was produced with medium volume in hydrolysis and fermentation processes of 1.5 and 3.5 ml/g, respectively. The efficiency of bioconversion processes not only depends on conditions used for cultivation but also depends upon the efficiency of strains used to utilize the lignocelluloses. UV mutagenesis of ***Streptomyces aegyptia*** was considered in this study. Results showed different effects among the lignocellulose enzymes measured in this study. Strain exposed to UV light (280-320 nm) at a distance of 10 cm for 20 seconds was the best one for sugar (57.3 mg/g) and bioethanol production 5.8 g/l (29 mg/g).

Keywords:
Bioethanol, manure, rice straw, solid-state hydrolysis and fermentation, ultraviolet (UV) mutagenesis.

1. INTRODUCTION

Nowadays, we face the depletion of energy resources, besides increasing human consumption. Moreover, the prices of fossil fuels in recent days are unstable. These problems motivate the development of alternative energy sources such as bioethanol. It is an environmentally friendly fuel

and can decrease greenhouse gas emissions **[1, 2].** Apart from food-based bioethanol, second-generation bioethanol was distinguished by its use of lignocellulosic biomass as raw materials in the production process **[3].** Egypt has large resources of lignocellulosic biomass from agricultural and animal wastes. Agricultural waste produced about 35 million tonnes annually, 40% of which is used for animal feeding, and the rest can be available for energy purposes. Currently, the most promising and abundant lignocellulosic biomass derived from plant residues and animal waste in Egypt is rice straw and manure, respectively **[4].**

Rice straw and manure conversion to ethanol can be carried out in submerged or solid-state mode. Solid-state hydrolysis and fermentation is a system in which the hydrolysis and fermentation processes have been occurring in a minimum amount of water that makes a moist solid mass. The growth and enzymatic activity of the microorganism occur in the liquid film on the surface of the solid particles substrate **[5].** It has been used for some advantages over the submerged mode: minimizing energy consumption, while maximizing product concentration, and less wastewater production **[6].** Solid-state systems for bioethanol production are practical for rice straw and manure utilization and have been used for this purpose in several studies **[7–12].**

Improving bioethanol production in a solid-state system is controlled by a large number of factors, each of which is critical for process development. These included the selection of microorganisms and substrates, and optimum culture process parameters **[5, 13]**. Medium volumes of the hydrolysis and fermentation processes have been considered critical process parameters in the solid-state systems for mass transfer of the water and salts across the microbial cells. The control of these parameters could be applied to improve the microbial activity and metabolic production of microorganisms **[14].**

One of the essential factors involved in the achievement of higher titers of bioethanol production is strain improvement **[15]**. Random mutagenesis and fermentation screening have been reported as effective way to improve the productivity of industrial microbial cultures **[16].** Ultraviolet (UV) irradiation is the most widely used mutagen which induces a broad spectrum of point mutations **[17].** The efficiency of selection is depending on several factors such as the type of culture, the mutagen dose and exposure time, and the conditions of treatment and post-treatment. Examples of stain improvements can be found in the actinomycetes cultures capable of over-producing lignocellulosic enzymes in quantities higher than the wild type **[18, 19].** As economic analyses indicated that the process of lignocellulose degradation contributes to 40–45% of the total bioethanol cost **[20].** Hence, bringing down these costs through strain improvement will be of prime concern to make lignocellulosic ethanol commercially viable.

In this study, the production of bioethanol from a mixture of rice straw and manure was reported under solid-state conditions using a co-culture of *Streptomyces aegyptia* and *Candida tropicalis*. In addition, medium volumes in hydrolysis and fermentation processes were optimized for maximum ethanol production using response surface methodology (RSM) methodology. Ultraviolet (UV) mutagenesis of *Streptomyces aegyptia* has been performed also to enhance lignocellulosic enzymes.

2. MATERIAL AND METHODS

2.1. Microorganisms

The actinomycete strain used in this study was previously isolated from rice straw compost **[21].** It was identified by the molecular method as *Streptomyces aegyptia* (Genebank accession number MT534590). Culture was maintained for 5 days on starch casein agar medium containing (g L^{-1}): soluble starch, 10.0; casein (vitamin-free), 0.3; KNO_3, 2.0; NaCl, 2.0; K_2HPO_4, 2.0; $MgSO_4.7H_2O$, 0.05; $CaCO_3$, 0.02; $FeSO_4.7H_2O$, 0.01.

The yeast strain used in this study was previously isolated from mango fruit **[21].** It was identified by the molecular method as *Candida tropicalis* (Genebank accession number MT874505). The cultures

used for inoculation were prepared by growing the organism on a rotary shaker at 120 rpm and 30 °C for 16 h, in yeast-peptone glucose (YPG) agar medium containing (g L^{-1}): yeast extract, 10; peptone, 20; glucose, 20.

2.2. Substrates

Rice straw was procured from an animal feed supplier of Suez Canal University (Ismailia, Egypt). The straw was sun-dried, cut as apiece of 1 cm, and stored in sealed bags at room temperature until use.

Buffalo manure was supplied from the farm College of Veterinary Medicine of Suez Canal University (Ismailia, Egypt). It was autoclaved immediately after collection at 121ºC for 30 min and dried in an oven at 70 ºC until dryness. Grinding was done using an electric mill grinder blender and the powdered manure (0.2 - 2 mm) was stored in the refrigerator until use.

2.3. Solid-state hydrolysis and fermentation

The process was carried out in Petri plates, each containing 1g manure and 0.5 g rice straw. The substrates used in this study were moistened with distilled water to keep them wet and sterilized for 15 min. The hydrolysis medium (KH_2PO_4 5 g/l, $MgSO_4$ 3.5 g/l, and Tween 7 ml/l) was added and inoculated with (**$1x10^7$**) equivalent CFU of *Streptomyces aegyptia* spore. The plates were incubated at 30 ºC for 3 days. Subsequently, the fermentation process was conducted on the same plates by adding fermenting medium (KH_2PO_4 3 g/l and $MgSO_4$ 4 g/l) solution and 5ml of *Candida tropicalis* culture (10^8 CFU /ml). All of the experiments were carried out in triplicate and the results were reported as the mean of these replicates.

2.4. Experimental design for optimization

A central composite design (CCD) and response surface methodology were used to optimize the most effective medium volume added to the substrate in hydrolysis (1- 4 ml/g) and fermentation (1- 4 ml/g) processes for maximum ethanol production in a solid-state system. Thirteen experimental runs were designed in the CCD with five center point experiments. The levels of each variable parameter are presented in Table (1). The design was represented by a second-order polynomial regression model to generate surface and contour plots:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{1 \le i \le j}^{k} \beta_{ij} x_i x_j + \varepsilon$$

Equation (1)

Where Y is the response of ethanol yield; x_i is process factors; β_0 is the offset coefficient; β_i is linear coefficients; β_{ii} is quadratic coefficients; β_{ij} is interaction coefficients and ε is the residual associated with the experiments. The experimental data analyses were performed using Minitab software version 19.1. All experiments were conducted in triplicate and the data were presented as the mean of these replicates.

2.5. UV Mutagenesis of *Streptomyces aegyptia*

The process was carried out in Petri plates, each containing 1 g manure and 0.5 g rice straw. The medium volume was adjusted by adding 1.5 ml of medium solution (KH_2PO_4 5 g/l, $MgSO_4$ 3.5 g/l, and Tween 7 ml/l). The plates were sterilized for 15 min. The medium was inoculated with *Streptomyces aegyptia* spores that were applied to UV irradiation. The spore suspension of *Streptomyces aegyptia* **$1x10^7$** equivalent CFU/ml was irradiated with UV light (280- 320 nm) for 0.3, 1, 5, 15, and 30 min, at a fixed distance of 10 cm. After the irradiation, to protect against

photoreactivation, the tubes were wrapped with aluminum foil and all these steps were carried out in a dark room**.** The plates were incubated at 30 ºC for 3 days. Subsequently, the fermentation process was conducted on the same plates by adding 3.5 ml of medium (KH_2PO_4 3 g/l and $MgSO_4$ 4 g/l) solution and 5ml of *Candida tropicalis* culture (10^8 CFU/ml). All trials were performed in triplicate and the responses were measured in terms of enzymes (Lignin peroxidase, xylanase, and endocellulase), sugar and ethanol production.

2.6. Analytical methods

2.6.1. Extraction preparation

A known amount of solid fermented substrates (0.2 g) was treated with 2 ml of distilled water and agitated thoroughly. After 10 min, the contents were centrifuged and the clear supernatant was used for analytical determinations.

2.6.2. Reducing sugar and ethanol determination

Reducing sugar ware determined by the 3,5-dinitro salicylic acid DNS method **[22]** and glucose was used as the standard. Ethanol was measured using tri-n-butyl phosphate (TBP) solvent extraction [**23**], and its content was estimated by the spectrophotometric method **[24]**. Ethanol yield was calculated using the following equation **[25]**:

$$\text{Ethanol yield (mg/g)} = \frac{\text{Final ethanol concentration (mg)}}{\text{Theoretical sugar in substrate (g)}} \quad \text{Equation (2)}$$

2.6.3. Enzymes assay

Endocellulase and xylanase activities were assayed according to the analytical procedure recommended by the international union of pure and applied chemistry (IUPAC) using 1 % (w/v) carboxymethylcellulose and 1% (w/v) birchwood xylan**,** respectively **[26]**. The reaction mixture, containing suitably diluted crude enzyme solution (0.25 ml) and 0.25 ml of substrate solution prepared in 50 mM citrate buffer (pH 5.0), was incubated at 30^{o}C for 30 minutes. In all the cases, after incubation, the released reducing sugar was estimated by the DNS method **[22].** The reducing sugar was estimated from the absorbance measured at 540 nm using glucose (for endocellulase) and xylose (for xylanase) as standards. One unit of enzymatic activity is defined as the amount of enzyme that releases 1 µmol reducing sugar/g/minute.

Lignin peroxidase assay was determined in a reaction mixture containing (final concentration) sodium tartrate buffer (125 mM, pH 3.0), Azure B (160 µM), H_2O_2 (200 µM), and 1.0 ml of crude enzyme. The reaction is initiated by adding 0.5 ml of H_2O_2. One unit of enzyme activity is equivalent to an absorbance decrease of 0.1 units/g/minute **[27]**.

3. RESULTS

In the present study, the rice straw mixed with manure was used as lignocellulose substrates for bioethanol production. A solid-state mode was applied for hydrolysis and fermentation processes to increase the concentration of ethanol products. Improving solid-state hydrolysis and fermentation in this experiment was applied through medium volume optimization and strain improvement of *Streptomyces aegyptia.*

3.1 Optimization of medium volume for improving bioethanol production

The effect of medium volume in the hydrolysis and fermentation processes on ethanol production was studied by response surface methodology (RSM). The design was applied in 13 experimental runs

with different combinations of two factors. The response along with the predicted response was given in Table (1). A maximum ethanol concentration of 4.4 g/l (22 mg/g) was produced with medium volume in hydrolysis and fermentation processes of 1.5 and 3.5 ml/g, respectively. Regression analysis of the ethanol yield data from Table (1) resulted in the following quadratic equation (3):

$$Y = 13.786 + 3.368\ A + 2.909\ B - 0.3848\ A*B - 0.0293\ B*B - 1.1131\ A*B$$

Equation (3)

Where Y is ethanol yield (mg/g), A is the medium volume in the hydrolysis process (ml/g) and B is the medium volume in the fermentation process (ml/g).

The ANOVA of the quadratic regression model was applied to evaluate the impact and significance of terms such as linear terms, squared terms, and interactions in the regression equation, and the results were summarized in Table **(2)**. The model exhibits a high determination coefficient ($\mathbf{R^2}$ = 97.9%), explaining 97.9% of the variability in the response, as well as a high value of the adjusted coefficient determination ($\mathbf{R^{2(adj)}}$ = 97.3%), suggesting a high adjustment of the theoretical response values to the experimental data by this model. A very low probability obtained from the regression analysis of variance (ANOVA) demonstrated that the model was significant. In this study, a factor of medium volume in the hydrolysis process was significant, as the p-values calculated for these factors were less than 0.05. Therefore, changes in this parameter (1- 4 ml/g) could significantly impact ethanol production. However, medium volume in the fermentation process was non-significant, as the p-values calculated for these factors were more than 0.05. Therefore, changes in this parameter (1- 4 ml/g) could non-significantly impact ethanol production. The lack of fit p-value of 0.507 implies that the lack of fit is not significant relative to the pure error. The non-significant lack of fit is positive because it demonstrates a good fit of the model to the data. A positive sign for the coefficient indicated that the tested variables had a synergistic effect on the ethanol yield while a negative coefficient indicated an antagonistic effect. The interaction between the two variables was observed to be significant at the 5% level. The degrees of freedom (DF) indicate the number of observations in the experiment that are free to vary while estimating statistical parameters. Thus, the degree of freedom for the model was 5. Adjusted sums of squares (Adj SS) in Table (2) quantified the amount of variation in the response data that is explained by each term in the model. The error sum of squares quantified the variation in the data that the predictors do not explain. Adjusted mean squares (Adj MS) measured how much variation a term or a model explains. Unlike the adjusted sums of squares, the adjusted mean squares consider the degrees of freedom. Minitab uses the adjusted sums of squares (Adj SS) and adjusted mean squares (Adj MS) to calculate the p-value for a term and calculate the adjusted R^2 statistic.

The effects of variables on ethanol yield were analyzed using contour and surface plots presented in Figure (1) based on Equation (3). An increase in the concentration and yield of ethanol was observed when the medium volumes were decreased in the hydrolysis process by *Streptomyces aegyptia* (1.5 ml/g) and increased in the fermentation process by *Candida tropicalis* (3.5 ml/g).

3.2. UV Mutagenesis of *Streptomyces aegyptia* for improving bioethanol production

The objective of UV mutagenesis in this study was to develop an improved *Streptomyces aegyptia* strain with higher titers of lignocellulosic enzymes for bioethanol production. Different doses of UV light exposure were applied to the spore suspension of *Streptomyces aegyptia*. UV light exposure for 20 sec (0.3 min) has resulted in a 1.27-fold increase (5.81 g/l) in bioethanol concentration as compared to that of the wild-type strain (4.57 g/l).

From the data mentioned in Table (3), mutant strains of *Streptomyces aegyptia* achieved different enzyme production patterns. Lignin peroxidase and endocellulase activities assessed the highest value

when exposed to UV light for 0.3 and 1 min respectively however, results showed that all UV exposure times didn't increase the production of xylanase but made a complete inhibition.

4. DISCUSSION

Rice straw and manure are common sustainable wastes on farms. Elimination of these wastes through burning or left to decompose naturally could have damaging effects on the environment. Converting rice straw and manure into biofuel sources such as bioethanol might reduce environmental problems and improve agricultural waste economics **[28].** The present study investigated the potential utilization of rice straw and cow manure for ethanol production through solid-state mode using the co-culture of *Streptomyces aegyptia* (for lignocellulose decomposition) and *Candida tropicalis* (for ethanol fermentation). In this respect, several studies focused on rice straw utilization for bioethanol production **[29, 30],** and produced bioethanol using pretreated dairy manure as a carbon and nitrogen source **[11, 25]**.

In this study, the solid-state mode was applied in hydrolysis and fermentation processes. This mode of conversion was suitable for *Streptomyces aegyptia* to degrade the lignocellulose substrates and for *Candida tropicalis* to ferment sugar to ethanol. Actinomycetes, especially *Streptomyces* strains are well known for their ability to decompose lignocelluloses and the hyphal growth mode of the employed filamentous actinomycetes gives them an easily penetrate inside the substrate in solid-state fermentation. In one study, isolation of actinomycetes was applied for degrading the rice straw and optimizing the enzymatic activity of *Streptomyces viridiochromogenes* in solid-state mode **[9]**, whereas another study used *Streptomyces psammoticus* for rice straw utilization in solid-state mode and optimized the fermentation process resulted in two-fold increased in enzymatic activity **[31]**. Yeasts from genera *Candida* are capable of fermenting both glucose and xylose into ethanol in solid-state fermentation **[5, 32]**. In one study, bioethanol from rice straw hydrolysate was produced using *Candida shehatae* in a level yield of 71% more than *Saccharomyces cerevisiae* NBRC 0224 **[33]**. Improving the ethanol production in solid-state fermentation could be performed through optimized medium volume and by increasing the strain efficiency **[16]**. That was similar to the design applied in one study in which *Aspergillus sp*. SU14 was treated with ultraviolet irradiation and optimized culture conditions for the production of cellulase using solid-state fermentation **[34]**.

In this study, five levels of medium volume were applied in the hydrolysis and fermentation processes. High medium volume (4 ml/g) in the hydrolysis process using *Streptomyces aegyptia* had a negative effect that may decrease substrate porosity, which in turn prevents oxygen penetration **[13].** However, low medium volume (1.5 ml/g) was preferred by *Streptomyces aegyptia* because manure considered itself a rich substrate with mineral salts that were sufficient for microbial growth and activity. A similar trend was observed in a study of xylanase enzyme production in *Streptomyces sp.* ESRAA-301097 used solid substrates and no enhancement occurred when the metal salts source was added **[35]**, whereas another study **[36]** showed that solid to liquid (1:2) was the best requirement for maximizing cellulose hydrolysis by *Aspergillus niger* under solid-state fermentation.

In the fermentation process, high medium volume (3.5 ml/g) was preferred by *Candida tropicalis***.** A similar observation was illustrated in one study in which ethanol yield was increased by increasing the liquid medium applied to solid substrates in the fermentation step and an ethanol yield of 179 mg/g was obtained with an optimum liquid to solid ratio of 9:1 **[14]**.

Besides the medium volume, improvement of the bioconversion process of lignocelluloses to ethanol depends also upon the efficiency of strains used. Strain improvements via UV mutagenesis are an important approach for increasing bioethanol production. UV mutagenesis of ***Streptomyces aegyptia*** was considered in this study for improving lignocellulose degrading enzyme production and thereby increasing bioethanol production.

Table (1): Central composite design for solid-state hydrolysis and fermentation of rice straw and manure showed ethanol concentration (g/l) and ethanol yield (mg/g).

Run NO.	Medium in hydrolysis (ml/g) **(A)**	Medium in fermentation (ml/g) **(B)**	**Ethanol concentration (g/l)**		**Ethanol yield (mg/g)**	
			experimental	predicted	experimental	predicted
1	2.5	2.5	4.0	4.0	20	20
2	3.5	1.5	3.8	3.9	19	20
3	1.5	3.5	4.4	4.4	22	22
4	2.5	2.5	3.9	4.0	20	20
5	2.5	2.5	4.0	4.0	20	20
6	2.5	1	3.9	4.0	20	20
7	1.5	1.5	3.9	3.9	20	20
8	2.5	2.5	3.9	4.0	20	20
9	4	2.5	3.4	3.4	17	18
10	3.5	3.5	3.4	3.4	17	17
11	1	2.5	4.2	4.2	21	21
12	2.5	2.5	4.0	4.0	20	20
13	2.5	4	4.0	4.0	20	20

Table (2): Analysis of variance for ethanol yield (mg/g) produced in solid-state hydrolysis and fermentation.

Term	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Model	5	42.8543	8.5709	182.51	0.000
Linear	2	30.4615	15.2308	324.33	0.000
Medium volume for hydrolysis (A)	1	30.4546	30.4546	648.51	0.000
Medium volume for fermentation (B)	1	0.0069	0.0069	0.15	0.706
Square	2	2.4813	1.2407	26.42	0.000
A*A	1	2.4530	2.4530	52.24	0.000
B*B	1	0.0142	0.0142	0.30	0.588
2-Way Interaction	1	9.9115	9.9115	211.06	0.000
A*B	1	9.9115	9.9115	211.06	0.000
Error	20	0.9392	0.0470		
Lack-of-Fit	3	0.1171	0.0390	0.81	0.507
Pure Error	17	0.8221	0.0484		
Total	25	43.7935			

Model Summary: R^2 = 97.9%; $R^{2\,(adj)}$ = 97.3%

Symbol; DF, degrees of freedom; SS, sum of squares; MS, mean sum of squares.

Results obtained after UV mutagenesis of ***Streptomyces aegyptia***, indicated that enzyme activities of lignin peroxidase and endocellulase under low UV exposure time (0.3-1 min) increased by 1.4 and 1.6-folds respectively as compared with the wild type strain then decreased drastically by further increased in time exposure.

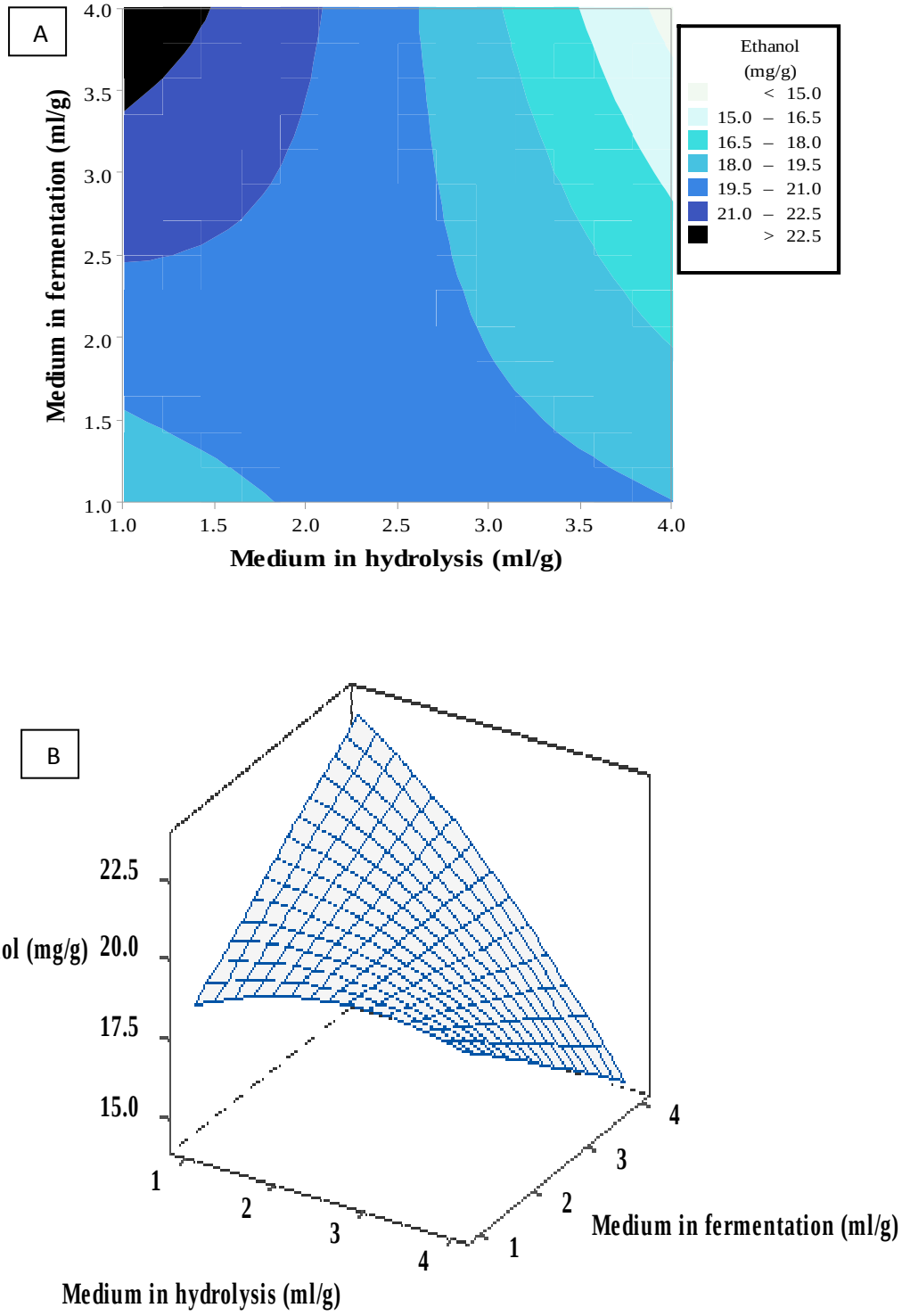

Figure (1): Counter (A) and surface (B) plots of two-way interactions of medium volume (ml/g) in hydrolysis and fermentation processes for maximal ethanol yield (mg/g).

Table (3): Effects of ultraviolet irradiation on the production of *Streptomyces aegyptia's* enzymes, sugar, and ethanol.

UV exposure time (min)	Lignin peroxidase (U/min.g)	Endocellulase (U/min.g)	Xylanase (U/min.g)	Sugar Conc. (mg/g)	Ethanol yield (mg/g)	Ethanol concentration (g/l)
0	0.4	1.3	3.25	54.8	20.5	4.1
0.3	0.56	1.6	0.0	57.3	29	5.8
1	0.32	2.1	0.0	29.7	13.5	2.7
5	0.24	1.5	0.0	9.6	4	0.8
15	0.08	0.9	0.0	9.5	3	0.6
30	0.0	0.0	0.0	9.0	0.5	0.1

The enhanced activities could be due to a mutation(s) in regulatory genes coding and controlling expression for these enzymes **[37]**. These results were in agreement with other studies, in which mutagenesis of *Streptomyces durhamensis* with UV radiation was performed and the cellulase activity of the mutant was increased to 1.86-fold compared to the wild strain **[38],** UV treatment of *Streptomyces griseoaurantiacus* improved endoglucanase activity by 57.4 % compared to the wild-type enzymes **[19]**, the selected mutant strain, *Aspergillus sp*. SU14-M15, produced cellulase in a yield 2-fold improved activity than that of the wild strain **[34]**, mutant strains from *Streptomyces sp.* were treated with ultraviolet light (245 nm) and two mutant strains were able to cause a higher percentage of lignin degradation than the wild type **[39]**. However, the UV exposure time used in this study showed a negative effect on the xylanase activity of the strain. That could be due to a mutation(s) in regulatory gene(s) coding xylanase enzyme resulting in preventing the expression of that gene **[37].**

These results were in agreement with a study that applied and exposed spore suspensions of *Streptomyces lydicus* to UV-irradiation (fixed at 25cm and 254 nm) at different time exposure intervals (5, 10, 15, and 20 min), and found that the lowest time exposure of UV radiation caused complete inhibition of laccase enzyme **[40]**. Thus, the effect of UV light exposed to ***Streptomyces aegyptia*** differs among the lignocellulose enzymes. That was similar to other results **[41],** that applied *Trichoderma reesei* to mutagenesis by UV radiation in a good way but couldn't successfully isolate one mutant that showed increased amounts of all components of the cellulase enzyme complex. The improved strain contains the highest frequency of desired mutations while minimizing the frequency of mutations that may offset the positive impact of the desired mutation. Strain exposed to UV light (280-320 nm) at a distance of 10 cm for 20 seconds was the best one for sugar (57.3 mg/g) and bioethanol production (29 mg/g). That result was good in comparison with other results **[39]** showed that UV radiation had different effects on lignocellulosic enzymes of *Streptomyces sp.* and these radiations did not enhance the organism's overall ability to degrade lignocellulosic biomass in comparison with the wild-type.

5. CONCLUSION

Bioethanol was produced from rice straw and manure by solid-state hydrolysis and fermentation using the co-culture of *Streptomyces aegyptia* and *Candida tropicalis*. The maximum ethanol concentration of 4.4 g/l (22 mg/g) was produced with medium volume in hydrolysis and fermentation processes of 1.5 and 3.5 ml/g, respectively. It can be opined that UV mutation of *Streptomyces*

aegyptia had become successful in increasing the sugar and bioethanol productivity. Strain exposed to UV light (280-320 nm) at a distance of 10 cm for 20 seconds was the best one for sugar (57.3 mg/g) and bioethanol production (29 mg/g).

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