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Fungal production of uricase on poultry wastes

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Abstract: The main objective of this study was the isolation of fungi inhabiting different sites of poultry wastes and find out the most active fungi in uricase production. Among the tested strains of fungi using the plate assay method, Trichoderma sp, Mucor spinosus, Aspergillus terrus and Aspergillus flavus were the most active fungal strains, which were able to produce uricase. These fungal strains were tested for uricase production under submerged fermentation conditions. The highest uricase activity in liquid medium containing uric acid was obtained from Aspergillus flavus (7.5 U/mL), Aspergills terreus (4.6 U/mL) and Trichoderma sp. (2.6 U/mL) . It was clear that Aspergillus flavus was the most active fungus and was selected for further studies. The optimum conditions for maximum A. flavus uricase production were at a temperature of 30°C, pH 6.5 ,sucrose concentration of 10 g /L and NH4NO3 concentration of 17.5 g/l. Purification of the enzyme was carried out to give the enzyme with greatest yield by comparing the enzyme activity with the total activity in the original extract

keywords: Fermentation, poultry wastes, Enzyme production, Optimization of uricase production, Aspergillus flavus.

1.Introduction

The poultry industry is currently facing several environmental problems. One of the major problems is the accumulation of alarge amount of wastes generated by intensive production which may pose disposal and pollution problems unless environmentally and economically sustainable management technologies are evolved [1-2]. Poultry manure contains a large and diverse population of viruses, bacteria, fungi and protozoa. Several fungi can utilize uric acid as a nitrogen source or satisfy their requirements of nitrogen and

carbon [3]. e.g *Aspergillus wentti*, *Trichoderma* sp and *Nocardia alba*. Microbial breakdown of uric acid in broiler litter is the major source of ammonia [4]. This decomposition process requires the presence of water and oxygen. Microorganisms commonly found in manure produced the enzymes uricase and urease that were specific to this reaction. Urate oxidase or uricase is an enzyme that catalyzes specifically the oxidation of uric acid to allantoin and plays an important part in nitrogen metabolism [5]. Various natural sources such as bacteria [6], fungi [7]and eukaryotic cells [8] were found to be uricase producers.

Aim of the Work: The aim of this study was planned to obtain an alternative method to utilize the poultry wastes which create enviromental pollution by using biological method .Also this study was extended to investigate

1- Isolation, purification and identification of some fungi grown on poultry wastes.

2- Screening the fungal isolates that has ability to produce uricase enzyme.

3- Optimization of some factors affecting fungal uricase production.

4- Extraction of uricase enzyme and recognition of its aplications.

2. Materials and methods

Sampling

The waste of chicken and duck were collected from different poultry farmes at Mansoura district from January 2019 to April 2019.

Isolation of Micro organisms

One hundred g of each poultry-waste sample was transferred into 15 cm diameter sterilized Petri-dishes containing 0.5g uric acid powder. The Petri-dishes were incubated at 30° C for one week. Serial dilutions from 10^{-1} to 10^{-3} were prepared using sterilized water [9]. One milliliter of each dilution was transferred to a sterilized plate containing uric acid induction medium as descriped before. Three dishes were used for each dilution for a particular media. All Petri dishes were incubated at 30 ° C for 4-7 days.

Identification of the isolated fungi

A morphological examination of species was first made with the naked eye and at the low magnification power of the microscope. Identification of isolated fungi was occurred by Light microscopy, which is ahelpful tool in studying fungi [10-11].

Preparation of inoculum

The selected uricolytic fungi were grown in slants of uric acid induction medium at 30° C for 4 days to which 10 ml sterilized water was added to prepare the fungal suspention .From this suspension, 1ml was transferred to 49 ml of submerged-fermentation medium and incubated for 4days at 30° C.

Production of crude uricase

The inoculated submerged fermentation media was filtered. Uricase activity was deterimined in both mycelium and filterate.The mycelium was collected and ground in a cooled mortar surrounded by an ice bath that acts as a cooling system. Glycine-Sodium Hydroxide Buffer pH 8.6 was used to facilitate grinding to form homogenous preparation .This preparation was then centrifuged at 3000 rpm for 15 minutes in a cooling system centrifuge in order to get rid of all insoluble particles. Finally, the clear supernatant was collected.

Preparation of substrate solution

One mg of uric acid was dissolved in 10 ml 0.1N NaOH and the pH waslowered to 7.2-7.4 with 0.1N HCL solution and the solution was completed to 100 ml with distilled water.The substrate was kept at 30°C to prevent crystallization of uric acid and was freshly prepared every day.

Uricase activity was determined spectrophotometrically.

a-Continuous assay: in two suitable cuvettes, to the first$_2$ add 3.0 ml of 20 mM boric acid buffer, 0.10ml of 3.57 mM uric acid solution and 0.02ml of uricase enzyme solution (test cuvette).In the second cuvette add 3.0ml of 20 mM boric acid buffer, 0.10ml of 3.57 mM Uric Acid Solution and 0.02ml of 20 mM Boric Acid Buffer. Immediately mixing was occurred by inversion and the decrease in A293nm was recorded for approximately 5 minutes. The ΔA293nm/minute was obtained using the maximum linear rate for both the test and blank.

b-Bradford protein assay

The protein content of the enzyme was determined according to [12]. 0.5 ml of the sample was mixed with 2.5 ml of protein reagent (Commasie blue reagent, G-250) and standing for 15 min at room temperature .There after, the optical density of the colour was measured against blank at 595 nm using Spectro UV-VIS RS spectrophotometer (Serial number :UV-VIS 0478; Labomed Inc.USA). The µg of protein was estimated using a standard curve of bovine serum albumin (BSA, Sigma).

Optimization of uricase production of *Aspergillus flavus* by using Box-Behnken design

For enlargement of uricase enzyme creation by the selected fungus, the response surface optimization was performed using the Box-Behnken design matrix **Table 1**. Accordingly, the independent variables were;Temperature, pH, Sucrose and NH_4NO_3 . The behavior of the three-factor system was explained by the following second order polynomial Eq

$$Y= \beta o + \sum \beta i Xi + \sum \beta ij XiXj + \beta ii Xi^2$$

Where:

Y = Predicted response, βo = Intercept term, βi= Linear effect, βij= Interaction effect, βii= Squared effect, Xi = Independent variable and Xj = Independent variable

The regression equation was generated using Design-Expert version 7 software by setting the optimum conditions that yield the maximum value of uricase. The total uricase in the filtrate was spectrophotometrically determined. The

statistical model was experimentally validated with respect to uricase production under the conditions predicted by the preceding model

Table 1: Box-Behnken experimental design method for screening of factors affecting on extracellular uricase enzyme production by *Aspergillus flavus*

Std	Run	Factor 1 A:PH Degree	Factor 2 B:Temp°c	Factor 3 C:Sucro seg/l	Factor 4 D:NH4NO 3 g/l	Uricase activity U/ml		Residus
						Actual uricase production	Predicted uricasepro duction	
4	1	7.50	40.00	10.00	17.50	2.5	3.10	-0.60
21	2	6.50	25.00	10.00	8.00	1.97	1.92	0.04
26	3	6.50	32.50	10.00	17.50	5.08	4.79	0.28
15	4	6.50	25.00	15.00	17.50	3.4	3.86	-0.46
7	5	6.50	32.50	5.00	27.00	4.7	4.29	0.40
23	6	6.50	25.00	10.00	27.00	5.65	5.21	0.43
19	7	5.50	32.50	15.00	17.50	4.63	4.09	0.53
8	8	6.50	32.50	15.00	27.00	2.22	2.77	-0.55
6	9	6.50	32.50	15.00	8.00	1.125	1.80	-0.68
1	10	5.50	25.00	10.00	17.50	0.8	0.47	0.32
22	11	6.50	40.00	10.00	8.00	7.36	7.61	-0.25
16	12	6.50	40.00	15.00	17.50	6.35	5.74	0.60
17	13	5.50	32.50	5.00	17.50	2.41	2.79	-0.38
5	14	6.50	32.50	5.00	8.00	3.54	3.26	0.27
27	15	6.50	32.50	10.00	17.50	5.14	4.79	0.34
20	16	7.50	32.50	15.00	17.50	1.1	0.53	0.56
25	17	6.50	32.50	10.00	17.50	4.16	4.79	-0.63
24	18	6.50	40.00	10.00	27.00	6.47	6.32	0.14
18	19	7.50	32.50	5.00	17.50	4.47	4.81	-0.34
9	20	5.50	32.50	10.00	8.00	3.27	3.02	0.24
2	21	7.50	25.00	10.00	17.50	6.89	6.72	0.16
13	22	6.50	25.00	5.00	17.50	3.32	3.83	-0.51
3	23	5.50	40.00	10.00	17.50	10.45	10.89	-0.44
10	24	7.50	32.50	10.00	8.00	1.915	1.54	0.37
11	25	5.50	32.50	10.00	27.00	3.03	3.31	-0.28
14	26	6.50	40.00	5.00	17.50	9.3	8.74	0.55
12	27	7.50	32.50	10.00	27.00	3.11	3.26	-0.15

3. Results and Discussion

Isolation of uricolytic fungi

Five uricolytic fungal genera and seven species belonging to three classes were isolated . These classes namely Ascomycetes, Hyphomycetes and Phycomycetes. Ascomycetes constituted the greatest number of all isolates which are represented by two genera

namely *Aspergillus* and *Pencillium*. WherePhycomycetes

Constituted the least number of isolates and represented by two genera namely *Rhizopus* and *Mucor*. The Hyphomycetes are represented by one genus namely *Trichoderma*. The highestnumber all over isolated genera was recorded by *A.favus* followed by *A.terrus* ,whereas, *Trichoderma* was the third genus in the count.The lowest count was recorded by *A.niger* followed by *Mucor*.

Determination of uricolytic production of the isolated fungi

The ability of isolated fungi for uricase production was detected by the plate method .The formation of clear zone around the fungal colony disc indicating its ability to produce uricase . The most active isolates were *Aspergillus flavus* and *Aspergillus terrus* while, *Trichoderma* sp and*Mucor spinosus* exhibited a lesser uricolytic activity.

Determination of uricolytic activity (unit /ml) of uricase producing fungi.

Table (2) shows the uricolytic activity of the isolates grown on a liquid uric acid medium .It is clear that *Aspergillus flavus* was the most

active fungi and was selected for further studies.

Table2: The extracellular uricolytic activity of isolates

Organism	Uricaseactivity(unit/ml)
Aspergillus flavus	7.5
Aspergills terreus	4.6
Trichoderma sp.	2.6

Optimization of factors affecting the production of uricase by *Aspergillus flavus* using Box-Behnken design

The relationship between the independent factors and the responses is determined by statistical analysis of the Box-Behnken data (**Table 1**). and the results are presented in **Table 4.** Among the studied variables ;temperature, sucrose and NH_4NO_3 were found to have a positive effect on sucrose production, while, pH was found to be negatively affected uricase production. The ANOVA indicates the model is significant. Values of " P-value " less than 0.0500 indicate model terms are significant [13]. In this case$_2$ B, C, D, AB, AC, BC, BD, A2, B2, C2, D2

are significant model terms. The model's goodness of fit was also checked by determination coefficient (R^2) , the adjusted R^2 and predicted R^2 values **(Table 3)** . The "Pred R-Squared" of 0.8287 is in reasonable agreement with the "Adj R-Squared" of 0.9303.The higher the adjusted R^2 the more accuracy of the relationships between the four variables (A,B,C, and D) and uricase production response. The coefficient of variation (CV) recorded 15.09, reflecting the high degree of reliability of the experiments that is usually indicated by the low value of CV. 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, pH is the only exception

Table 3. The Adjusted R- Squared" and the Predicted R-Squared

Std. Dev.	0.64	R-Squared	0.9678
Mean	4.24	Adj R-Squared	0.9303
C.V. %	15.09	Pred R-Squared	0.8287
PRESS	26.11	Adeq Precision	21.877

Table 4. Regression statistics and ANOVA for the Box-Behnken design experimental resuits

Soure	Sum of Squares	Df	MeanSquare	FValue	p-value Prob > F	
Moel	147.51	14	10.54	25.8	<0.001	Signifit
A-PH	1.77	1	1.77	4.33	0.0596	
BTp	34.68	1	34.68	84.93	<0.001	
CSucrose	6.62	1	6.62	16.22	0.0017	
DNH4N3	3	1	3	7.35	0.0189	
AB	49.28	1	49.28	120.68	<0.001	
AC	7.81	1	7.81	19.13	0.0009	
AD	0.51	1	0.51	1.26	0.2835	
BC	2.3	1	2.3	5.62	0.0353	
BD	5.22	1	5.22	12.79	0.0038	
CD	1.06E3	1	1.06E3	2.59E3	0.9603	
A^2	5.23	1	5.23	12.82	0.0038	
B^2	11.92	1	11.92	29.19	0.0002	
C^2	2.94	1	2.94	7.19	0.02	
D^2	5.52	1	5.52	13.52	0.0032	
Residual	4.9	12	0.41			
Lack of Fit	4.3	10	0.43	1.42	0.4817	not significat
Pure Error	0.6	2	0.3			
Total	152.41	26				

Discussions

Five genera were isolated from different samples collected from different poultry farms at Mansoura district. The results indicate that *Aspergillus flavus* uricase activity was found to be 7.5 U/ml, *Aspergills terreus* was 4.6 U/ml

and *Trichoderma* sp. was 2.6 U/ml under submerged fermentation in liquid uric acid medium. El-Weshy et al ., (2018) showed that *Aspergillus flavus* uricase activity was 10.91 U/mL. Geweely and Nawar (2011) illustrated that the maximum *Aspergillus niger* uricase

production was 47.40 U/mL. Also, Mabrouk et al. (2010) found the most activity of uricase produced by *Gliomastix gueg* (NRC 1A) was 275.98 U/mL.

The optimum fermentation condition (temperature, pH, sucrose and ammonium nitrate concentration) for uricase production by *Aspergillus flavus* were examined.The optimum pH for *Aspergillus flavus* uricase production was 6.5. This result is agreed with those obtained by Tohamy and Shindia (2001) who illustrated that the optimum pH was 6.0 for the production of uricase by *Aspergillus flavus* . However, this result is in disagreement with Ammar et al. (1988) who reported optimum pH for uricase by *Aspergillus flavus* was 9.2

Also, the temperature is considered one of the factors affecting uricase production. The present result showed that the optimal temperature for uricase production by *Aspergillus flavus* was 40°C .Yazdi et al. (2006) proved that 30°C was the optimal temperature to produce uricase by *Mucor hiemalis* and Abdel-Fattah and Abo-Hamed (2002) showed that the optimal temperature for the production of uricase was 30°C.

The influence of different values of sucrose on the production of uricase was carried out. The current result showed that the optimal concentration of sucrose for uricase production by *Aspergillus flavus* was 10 g/l.

The present results showed that the addition of NH_4NO_3 to the culture medium of production of uricase enzyme by *Aspergillus flavus* increases the production of enzyme and the optimum concentration of NH_4NO_3 was 17.5 g/l. This result is in disagreement with results obtained by El-Arini (1991) and Abo Dobara (1994).

Conclusion

The main objective of this study was the isolation of fungi inhaibiting different sites of poultry wastes and find out the optimum conditions for uricase production by some fungi isolated from poultry waste. Purification of the enzyme and determination of its physico-chemical properties are carried out. Five genera were isolated from different samples collected from different poultry farmes at Mansoura district.

Based on the above results, *Aspergillus flavus* can be considered as asource for the production of extracellular uricase that can be used in different applications. The optimization studies were carried to identify culture conditions that would improve uricase production by *A. flavus.* The result showed that the optimum temperature for fungal growth and uricase production by *Aspergillus flavus* was 40 °C .the maximum production of uricase and biomass yield were recorded at pH 6.5.

The fungal biomass and the enzyme yield were achieved in the medium supplemented with 10 g/l sucrose as a carbon source for *Aspergillus flavus* .Addition of NH_4NO_3 with 17.5 g/l to the media as a nitrogen source give the maximum production of uricase

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