A Study on the factors affecting biomass formation by a highly kojic acid producer fungal isolate from sugarcane molasses

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Abstract

Nowadays, microbial production of natural products represents a hot spot point in our environment and becomes an alternative way to chemical synthetic. In our research, *Aspergillus flavus* No**.** 3 shows great power in both biomass and kojic acid production. Plackett–Burman design utilize Egyptian sugarcane molasses as a sole carbon source giving kojic acid production of 0.82 (predicted 1.04) g/l to 24.65 (predicted 23.74) g/l, consuming sugar at 27.33 (predicted 26.96) % to 89.87 (predicted 87.46) % and forming dry biomass between 3.6 (predicted 3.8) g/l and 28.2 (predicted 28.05) g/l. The maximum kojic acid (24.65 g/l) and biomass value (28.2 g/l) obtained at 25°C; 9, 5 days of incubation, pH 3, 5; 0.5%, 2% inoculum size and the shaking rate at 150 rpm using fermentation medium (g/l) of sugarcane molasses, 60; yeast extract, 7, 3; KH_2PO_4, 2; $ZnSO_4.7H_2O$, 0.001 and $MgSO_4.7H_2O$, 1, respectively.

Keywords: Kojic-acid; Biomass; Aspergillus flavus; Sugarcane molasses, Optimization conditions; Plackett-Burman Design.

Introduction

Kojic acid (5-hydroxy-2-hydroxymethylgamma-pyrone) is a major secondary metabolite produced from carbohydrates by a variety of microorganisms; including *Aspergillus oryzae*, *A. flavus*, and *A. tamarii*, as well as same *Penicillium* species and certain bacteria (**Bentley, 2006 and Hazzaa *et al.*, 2013**). It can be produced by microorganisms using different carbon and nitrogen sources, also using agriculture based wastes under aerobic fermentation strategies.

Kojic acid is used in medical field as painkiller and an anti-inflammation drug (**Anonymous, 1992).** In food industry, it is used as a precursor for flavor enhancers and an anti-melanosis (blackening) of agriculture products during post- harvest by inhibiting polyphenol oxidase (**Chen *et al.*, 1991).** Kojic acid is also used as an ingredient for whitening agent and a protective against UV light in cosmetics (**Ohyama and Mishima, 1990).**

Kojic acid fermentation can be divided into two phases; growth phase and production phase (**Ariff *et al.*, 1996).** During growth phase, enzymes relevant to kojic acid metabolic pathway are produced and kojic acid is synthesized by the direct conversion of glucose through the multistep reactions of these enzymes without any cleavage into small fragments (**Arnstein and Bentley, 1953**). The cell bound enzymes system involved in kojic acid biosynthetic pathway was very stable when the cells were re-suspended in buffered glucose solution (**Bajpai *et al.*, 1982; Ariff *et al.*, 1996**).

Molasses are a by-product of sugar production from the sugar beet or sugar cane. Egyptian sugarcane molasses contain about 54-55 % as total sugar (glucose, sucrose, and fructose), 0.46 % as total nitrogen in addition to detectable amounts of some vitamins such as riboflavin and thiamin **(Mohamed *et al.*, 2012)**. Microelements in molasses are very important to the growth of microorganisms and their absence in cultural medium may cause lowering of biomasses formation **(Sobkowicz, 1997).** Sugarcane molasses was tested for kojic acid production by many researchers **(El-Aasar, 2006 and El-Kady *et al.*, 2014)**. **El-Aasar (2006) also** reported that the highest production of kojic acid by *A.parasiticus* was obtained from 60 g/L beet molasses with yield reached to 0.35 kojic acid g/g molasses. **El-Kady *et al*. (2014)** produced 15 g/L of kojic acid from sugar cane molasses using *Aspergillus flavus*.

Optimization of processing parameters plays an important role in the development of any process owing to their impact on the economy and efficacy of the process. Designing an appropriate production medium and conditions is of crucial importance to improve the efficiency and productivity of both microbial biomass and their bioactive metabolites, because it can significantly affects product concentration, yield, and the ease and cost of downstream product separation. A classical method of optimizing the fermentation conditions and medium constituents depends on single parameter whilst all the other factors are maintained at fixed levels. However, statistical planned experiments effectively explained the interaction of parameters and minimize the error in determining the effect of parameters (**Xu *et al.*, 2003 and Lakshmi *et al.*, 2011**). Also, the design of experiment reduces the number of experiments and increases process efficiency (**Chen *et al.*, 2009 and Senthilkumar *et al.*, 2012**).

Gupta *et al.* (1970) found that the toxigenic *A. flavus* strain formed less kojic acid than did the non- toxigenic strain in a glucose– salts medium. Also, Kojic acid can be produced either by using aerobic batch fermentation (**Madihah *et al.*, 1996**) or re-suspended cell material in buffered solution containing only glucose **(Ariff *et al.*, 1997**). Therefore, in this study we used Plackett–Burman design as a statistical method to optimize the nutritional and environmental conditions for dry mass formation from Egyptian sugarcane molasses by a non- toxigenic and highly kojic acid producer isolate of *Aspergillus flavus*.

Material and methods

1. Fungal isolate and culture conditions

Non-toxigenic *Aspergillus flavus* isolate No 3 which proved previously highly producer of kojic acid and biomass as described in our previous research **(Zohri *et al*., 2018).** Modified Czapek's dextrose liquid medium was

used for cultivation of this fungal isolate (**Ariff *et al.*, 1996).** The experiment was incubated at 28±1°C for 7 days on a rotary shaking (150 rpm) in three replicates. Biomass was collected and determined by using filtration while kojic acid and consumed sugar were determined in the supernatant.

2. Optimization using Plackett-Burman design

A sample of Egyptian sugarcane molasses from Abo-Qorqas sugar factory, El-Minia, Egypt, was collected and used as a kojic acid production medium in this study. Plackett-Burman design was used with eleven trails to test the effect of culture conditions and medium constituents on biomass formation, kojic acid production and sugar consuming from Egyptian sugarcane molasses (**Plackett and Burman, 1946).** The design was prepared according to **Zohri *et al*. (2018)** using Sigma XL program (Version 6.12).

3. Analytical analysis

Dry mass determined by filtrate the fungal mycelium through dried and weighed filter paper, washed three times with distilled water and then dried in hot air oven at 70 °C overnight. Kojic acid was determined spectrophotometrically at 540 nm (**Bentley, 1957; 2006; Liu *et al.*, 2016).** The residual sugar was analyzed spectrophotometrically according to **Dreywood (1946).** All analytical samples measured with T60 UV with a split beam UV visible spectrophotometer cover a wavelength range of 190-1100 nm.

Results and discussion

1. Kojic acid & biomass production by *A. flavus* No 3

On the modified Czapek's dextrose liquid medium containing 100g/l glucose as sole carbon source, *A. flavus* No 3 produced kojic acid at 10.58±0.01 g/l and 6.1±0.53 g/l dry mass **(Table 1)**. Glucose was used in screening of kojic acid producing *Aspergillus* species, since it is the simplest

sugar by **El Kady *et al.*, (2014). Rosfarizan and Ariff (2007)** recorded that glucose gave the highest kojic acid yield based on carbon consumed (0.365 g/g) followed by sucrose (0.279 g/g), starch hydrolysate (0.212 g/g) and fructose (0.195 g/g). **Hussani (2013)** reported that the highest Kojic acid concentration produced by *A. flavus* NSH9 was 15.38 g/l and recorded in presence of 110 g/l glucose in fermentation medium with pH value 4. **Rasmy and Basha (2016)** found that *A. oryzae* 124A produced 16.818 ± 0.006 g/l kojic acid by using glucose as carbon source.

Table (1) : Kojic acid and dry mass production (g/ l) by *Aspergillus flavus* No.3 using the modified Czapek's dextrose liquid medium.

Organism	Kojic acid (g/l ± SD)	Productivity (g/l/day)	Dry mass (g/l ± SD)
***A. flavus* No 3**	10.58 ± 0.01	1.51	6.1±0.53

2. Statistical optimization for biomass and KA production using Plackett-Burman design

In Plackett-Burman design each parameter was studied at two levels (-1 & +1). The results obtained indicated that there was a wide variation in kojic acid production of 0.82 (predicted 1.04) g/l to 24.65 g/l (predicted 23.74), consuming sugar 27.33 (predicted 26.96) to 89.87% (predicted 87.46) and dry mass varied between 3.6 (predicted 3.8) and 28.2 g/l (predicted 28.05). These results appeared the important effect of both medium components and environmental factors on the KA production and fungal biomass. The results also cleared a negative relationship between consuming sugar and biomass production consequently kojic acid production.

All the predicted values of Plackett-Burman design were located in close proximity to experimental values. Three-dimensional response surface

curves were generated to study the interaction between each two variables (Figures, 1 -4). The ANOVA results are shown in Table 2, the Model F values of KA, DM and consuming sugar are significant ($p<0.05$).

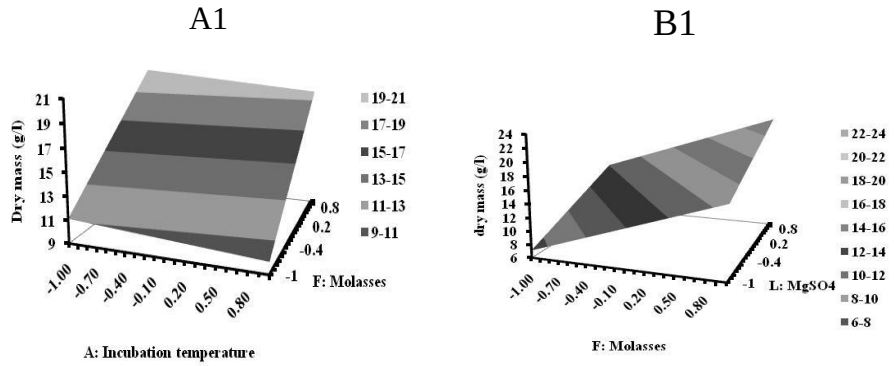
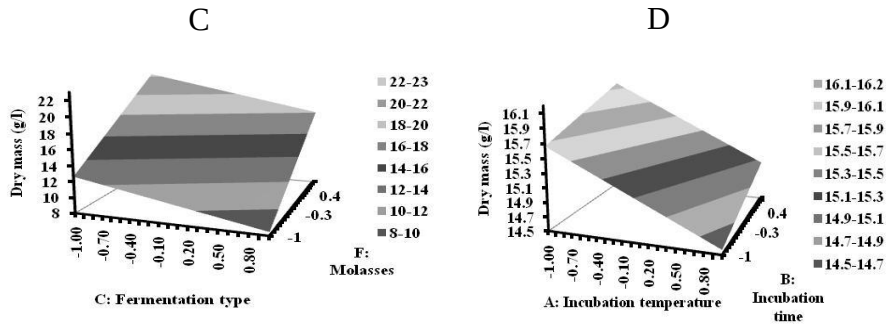


Fig. 1



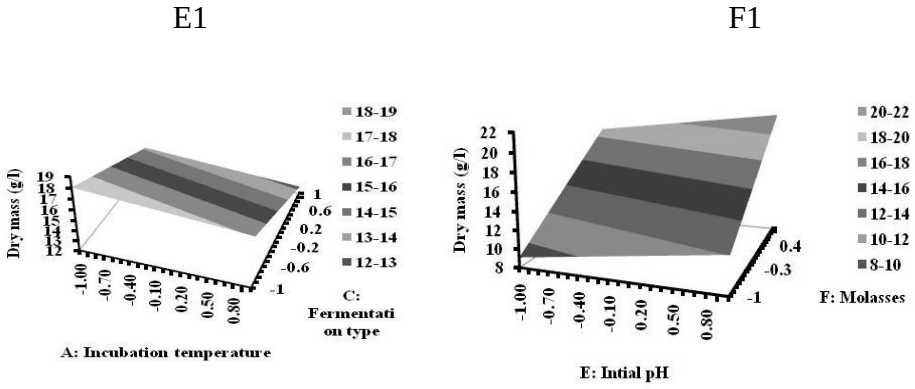
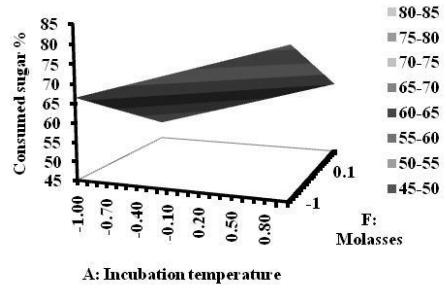


Figure (1): Response surface plots of Dry mass production by *Aspergillus flavus* No 3 showing the effect of two variables (other variables were kept at zero in coded unit) : (A1) Molasses and Incubation temperature, (B) Molasses and $MgSO_4$, (C) Molasses and fermentation type, (D) Incubation

temperature and incubation time, (E) Incubation temperature and Fermentation type, (F) Molasses and initial pH.

A



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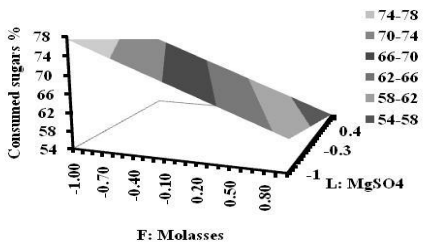

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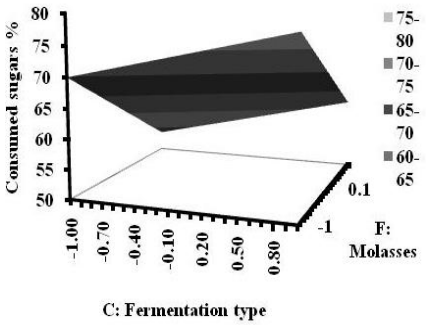

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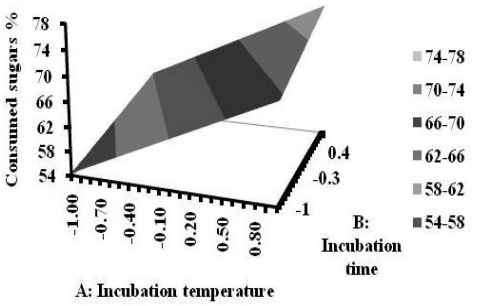

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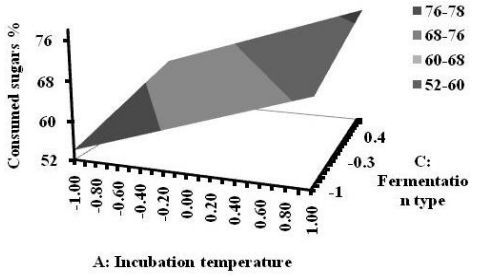

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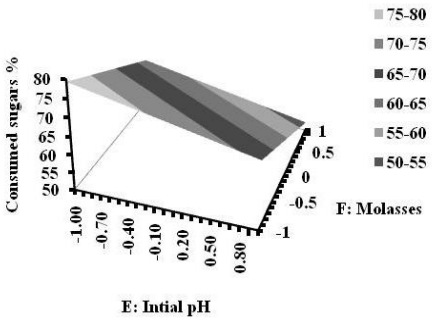

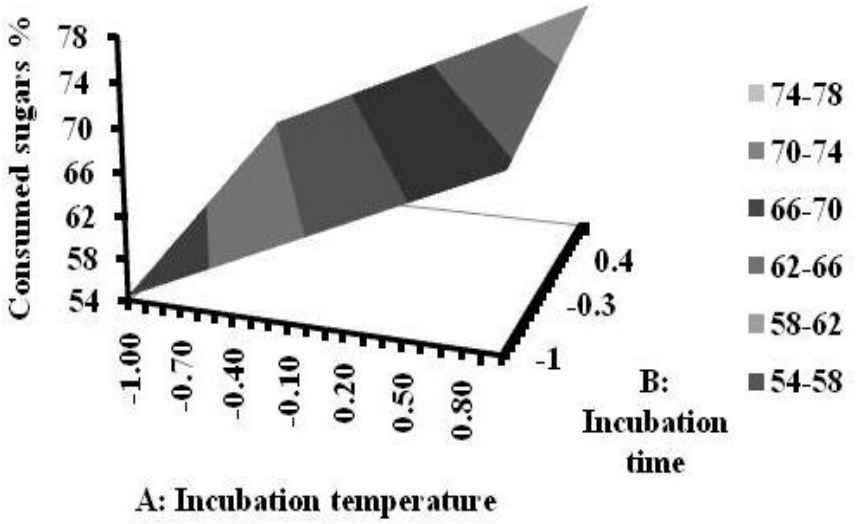

Figure (2): Response surface plots of consuming sugars % by *Aspergillus flavus* No .3 showing the effect of two variables (other variables were kept at zero in coded unit) : (A2) Molasses and Incubation temperature, (B) Molasses and $MgSO_4$, (C) Molasses and fermentation type, (D) Incubation temperature and incubation time, (E) Incubation temperature and Fermentation type, (F) Molasses and initial pH.

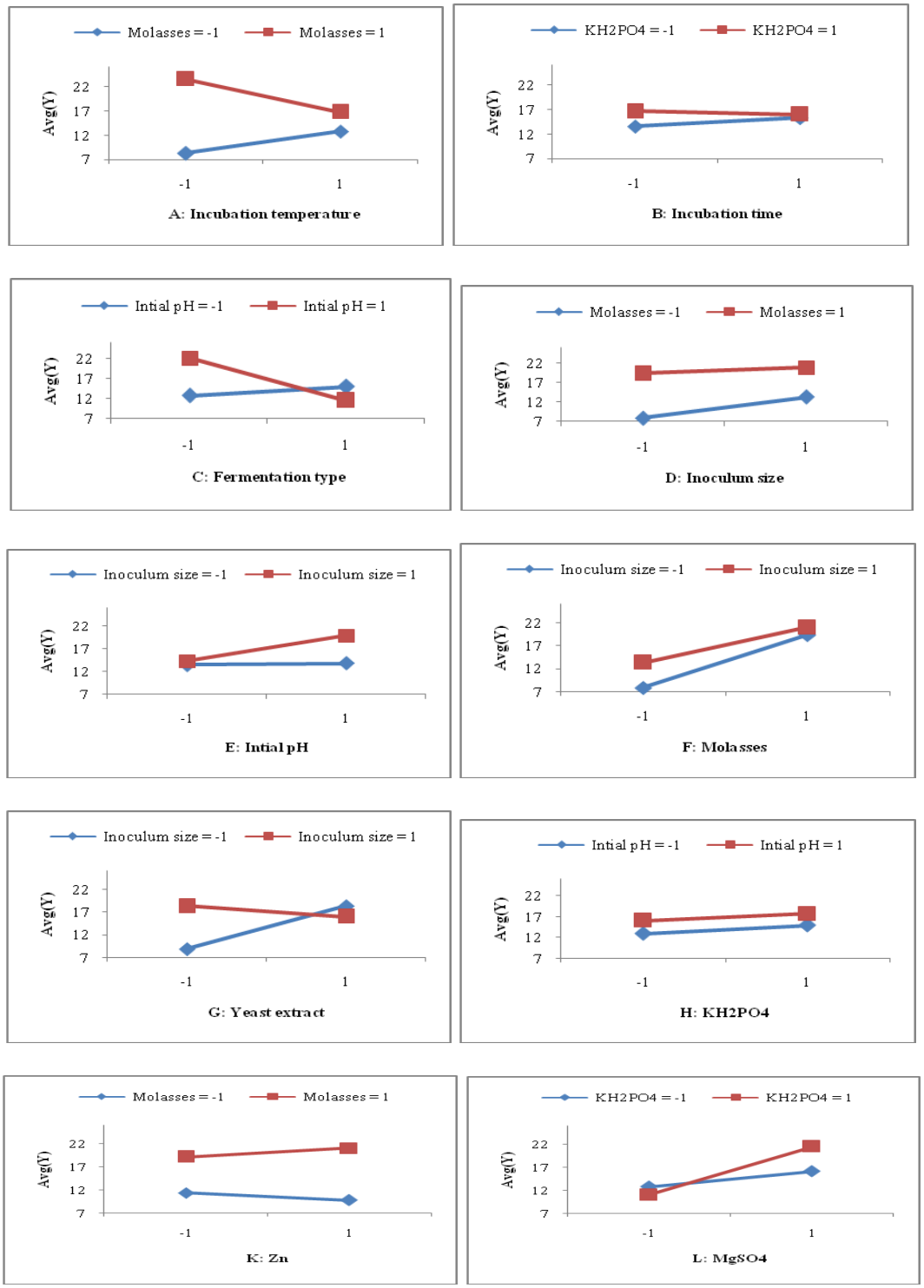

Figure (3): Main effects of different parameters on dry mass by *Aspergillus flavus* No. 3 showing effect of two variables (other variables were kept at zero in coded unit).

Table (2): Analysis of variance (ANOVA) of the results of kojic acid, consuming sugars and biomass production under eleven

Kojic acid (g/l)					
Source	**Degree of freedom**	**Sum of square**	**Mean square**	***F* value**	***P* value**
Model	**11**	**1167.6**	**106.15**	**197.79**	**<0.0001**
Error	**12**	**6.440**	**0.54**		
Pure Error	**12**	**6.440**	**0.54**		
Total (Model + Error)	**23**	**1174.0**	**51.05**		
Dry mass (g/l)					
Model	**11**	**1176.9**	**106.99**	**207.74**	**<0.0001**
Error	**12**	**6.180**	**0.515**		
Pure Error	**12**	**6.180**	**0.515**		
Total (Model + Error)	**23**	**1183**	**51.437**		
Consumed sugars %					
Model	**11**	**6571.8**	**597.44**	**44.095**	**<0.0001**
Error	**12**	**162.59**	**13.549**		
Pure Error	**12**	**162.59**	**13.549**		
Total (Model + Error)	**23**	**6734.4**	**292.80**		

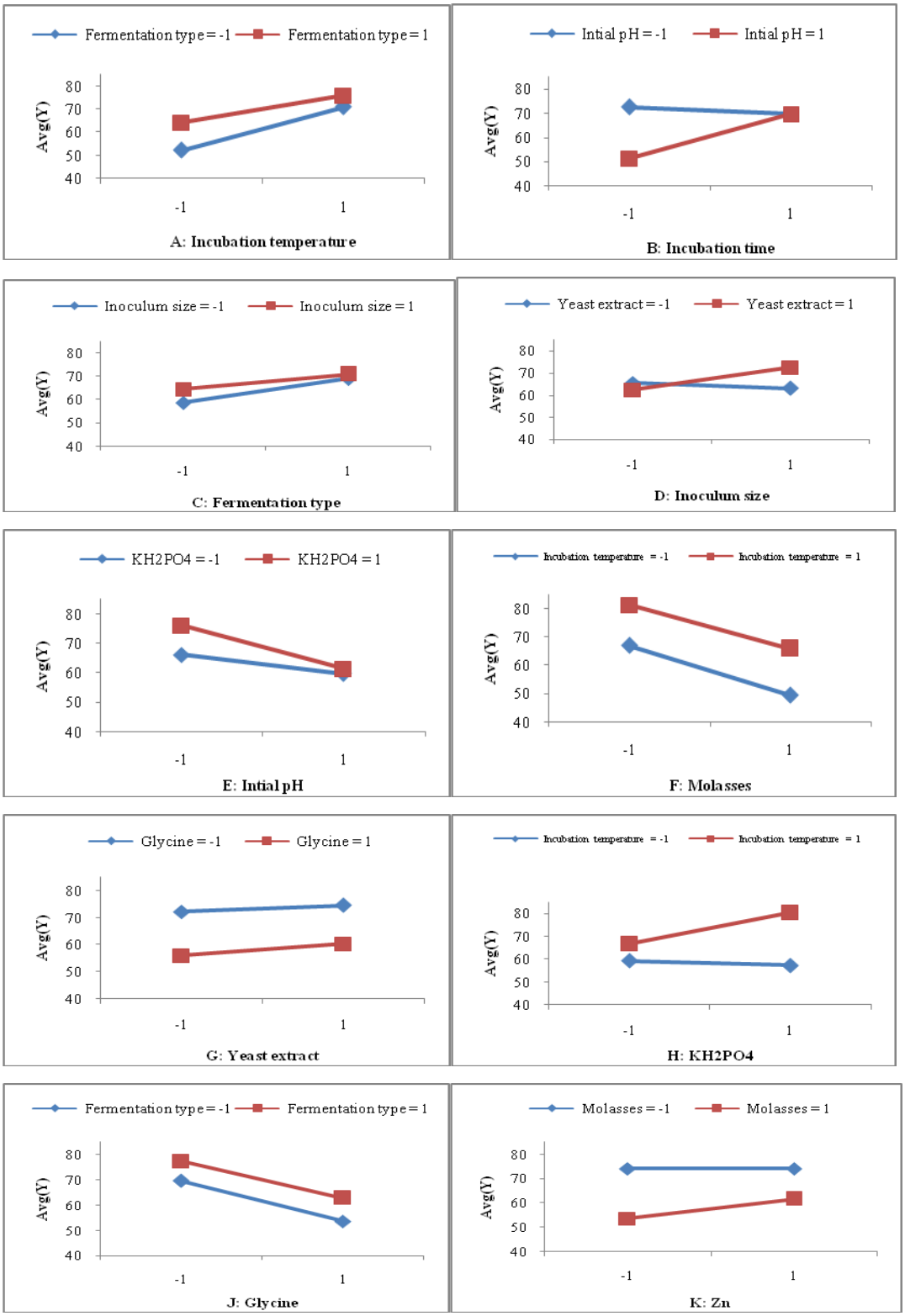

Figure (4): Main effects of different parameters on consuming sugars by *Aspergillus flavus* No 3 showing effect of two variables (other variables were kept at zero in coded unit).

Maximum biomass production (28.2 g/l) by *A. flavus* No. 3 was obtained under the fermentation conditions: Incubation temperature 25°C, incubation time 5 days, pH 5, inoculum size 2%, shaking rate at 150 rpm and medium constituents: Cane molasses 60 g/l, yeast extract 3 g/l, KH_2PO_4 2 g/l, $ZnSO_4.7H_2O$ 100 µg/l, glycine 0 µg/l and $MgSO_4.7H_2O$ 1 g/l. Maximum consuming sugars (89.87%) by *A. flavus* No 3 was obtained under the fermentation conditions: Incubation temperature at 35°C, incubation time 9 days, pH 3, Inoculum size 2%, shaking rate at 150 rpm and medium constituents: cane molasses 20 g/l, yeast extract 3 g/l, KH_2PO_4 2 g/l, $ZnSO_4.7H_2O$ 100 µg/l, glycine 100 µg/l and $MgSO_4.7H_2O$ 0.1 g/l. Maximum kojic acid production (24.65 g/l) by *A. flavus* No. 3 obtained under the fermentation conditions: Incubation temperature at 25°C, incubation time 9 days, pH 3, inoculum size 0.5%, shaking rate at 150 rpm and medium constituents: Cane molasses 60 g/l, yeast extract 7 g/l, KH_2PO_4 2 g/l, $ZnSO_4.7H_2O$ 100 µg/l and $MgSO_4.7H_2O$ 1 g/l.

Most studies conducted on the effects of culture pH towards the fungal growth and production of kojic acid was based on the initial culture pH **(Clevstrom and Ljunggren, 1985).**The maximum kojic acid production was achieved at pH 3.08 when ammonium nitrate was used as the nitrogen source **(Kitada *et al.*, 1967).**

The growth rate of mycelia reached their maximum 21.63 and 21.32 g L^{-1} at 11 days of cultivation in static cultures then gradually decreased, respectively (**El-Aasar, 2006)**. **Hassan *et al*. (2014)** found that a strain of *A. flavus* produced 34.8 g/l kojic acid and 10.7 g/l dry mass at pH 6.0 and 30°C after 16 days of incubation. **Kumar and Jayalakshmi (2017)** recorded that the maximum kojic acid production was 53 g/l and achieved at 30°C, while the highest dry mass yield was 14 g/l with fed-batch fermentation and appeared at 25°C. Maximum level of kojic acid obtained by **El-Kady *et al.***

(2014) using *A. flavus* No 24 was 53.5 g/l and recorded after eight days of incubation**.**

Hazzaa *et al.* (2013) found that *A. oryzae* var. *effusus* NRC14, *A. flavus* NRC13, *A. tamarii* NRC18 and *A. parasiticus* produced 0.602, 0.305, 0.288 and 0.088 g kojic acid /g glucose consumed, with formation of fungal biomass as 7.4, 6.4, 7.0 and 7.5 g/L, respectively, by using static cultivation. **Devi *et al.* (2016)** examined several soil fungi for kojic acid production and found that the maximum accumulation of kojic acid crystals was 16.8 g/l took placed by using coconut water as fermentation medium. They, also, reported that the yield of kojic acid reached to 47g/l by *Aspergillus flavus* FJ537130 at initial pH 7.0, 28ºC, after 16 days in fermentation medium had 4 g/l peptone, 0.1 g/l $MgSO_4$ and 1.0 g/l KH_2PO_4. **Yan *et al.* (2014)** found that the maximum concentration of kojic acid accumulated at the end of the fermentation was 33.1 g/l with yield based on sugar consumed and productivity of 0.36 g/g and 0.17 g/l/h, respectively.

Conclusion

The results obtained in this study appeared the possibility of using Egyptian sugarcane molasses as suitable substrate for kojic acid production by *Aspergillus flavus.* High production levels of kojic acid (24.65 g/l) and biomass (28.20 g/l) from 60 g sugarcane molasses were noticed at optimum nutritional and environmental conditions.

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الملخص العربي

دراسات علي العوامل المؤثرة علي انتاج الكتلة الحيوية بواسطة الفطريات المنتجة لأعلي كمية من حمض الكوجيك المنتج من قصب السكر المصري

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تمثل المنتجات الطبيعية المنتجة بواسطة الكائنات الدقيقة نقطة هامة للبيئة واصبحت طريقة بديلة للمنتجات الكيميائية. في هذا البحث اظهرت العزلة رقم 3 والتابعة لاسبرجلليس فلافس قدرة كبيرة علي انتاج حامض الكوجيك والكتلة الحيوية. وقد اظهر استخدام برنامج بلاكت برمان كبرنامج احصائي امكانية التهيئة المثلي لظروف انتاج الحامض باستخدام مولاس قصب السكر المصري كمصدر كربوني وحيد حيث اثبت ان كمية انتاج حامض الكوجيك المنتجة وصلت الي 24.65 جرام لكل لتر ووزن الكتلة الحيوية الجافة وصل الي 28.2 جرام لكل لتر باستهلاك كمية من السكر المستهلك وصلت الي 89.87 % من السكر المستخدم في تنمية الفطر. وقد سجل اعلي وزن للكتلة الحيوية الجافة للفطر عند درجة حرارة 25 درجة مئوية بعد مدة تحضين 5 ايام وكان الاس الهيدروجيني للوسط الغذائي5حجم اللاقحة 2% وباستخدام الحضان عند مستوي 150 لفة في الدقيقة والوسط الغذائي كان يحتوي علي 60 (جم / لتر) من مولاس قصب السكر و3 جرام من مستخلص الخميرة و2 جرام من ثنائي فوسفات البوتاسيوم و .001 جرام من كبريتات الزنك المائية و1جرام من كبريتات الماغنسيوم المائية .