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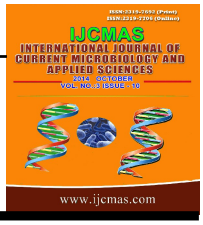

Original Research Article

Optimization Study for the Production of Kojic Acid Crystals by *Aspergillus oryzae* var. *effusus* NRC 14 Isolate

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Keywords

Aspergillus oryzae var. *effusus* NRC 14**,** Kojic acid crystals, Biomass, Carbon and nitrogen sources, temperature.

A B S T R A C T

The present study describes the optimal conditions for the production of kojic acid crystals by *Aspergillus oryzae* var. *effusus* NRC 14 isolate. The strain was subjected to different concentrations of glucose salts media, pH, temperature, carbon sources, nitrogen sources, medium volume and age and size of inoculum. The results concluded that the organism produces kojic acid with a maximum yield (49.5 g/l) and biomass dry weight 7.1 g/l at the 12th day of growth pH 4.0 and temperature 30ºC. Glucose and ammonium nitrate in concentrations of 100 g/l and 1.125 g/l,respectively were the favorable factors for kojic acid and biomass dry weight production (50 g/l kojic acid, 7.5 g/l biomass dry weight). On the other hand, 50 ml of culture medium and 10^6 spores/ml of inoculum from six days of incubation were found to be the best volumes for kojic acid production.

Introduction

Kojic acid (5-hydroxy-2-hydroxymethyl-gamma-pyrone; KA) is a major secondary metabolite produced from carbohydrates by a varietyof microorganisms, including *A. oryzae*, *Aspergillus flavus*, and *Aspergillus tamarii*, as well as *Penicillium* species and certain bacteria (Hazzaa et al., 2013; Ana Paula et al.2011; Bentley, 2006). Kojic acid can be produced in ample amount by using different carbon and nitrogen sources, also using agriculture based waste under aerobic fermentation strategies. To date, glucose has been described as a high kojic acid yielding raw material. However, use of some mutated strains of *A.flavus* has shown good yield of kojic acid by using potato, sago and corn starch etc. (Chaudhary et al. 2014). Kojic acid has several economic uses in various fields. In medical, kojic acid is used as an anti-bacterial and anti-fungal agent. In chemical industries it has been successfully used to make azo-dyes and biodegradable compounds. In food industries, kojic acid is used as anti-speck and anti- melanosis (blacking of product) agent for agricultural products. In addition, kojic acid is also used as a chelating agent and activator in insecticide production. Recently, a new application of kojic acid is

found in the cosmetic industry (Rosfarizan et al. 2010).

The present investigation describes the optimal conditions for kojic acid crystals production by *Aspergillus oryzae* var. *effusus* NRC 14 Isolate**.**

Materials and Methods

Mold culture

In a previous work (Hazzaa et al., 2013) *Aspergillus oryzae* var. *effusus* NRC14 was isolated from Egyptian soil and identified by the Regional Center for Mycology and Biotechnology, Al-Azhar Univ., Cairo, Egypt based on hyphal morphology and colony characters using an image analysis system.

Culture conditions

The strain was maintained on potato dextrose agar (PDA) slants at 4ºC and sub-cultured at intervals from 15-30 days.

Culture medium

Glucose salts liquid medium (May et al. 1931) was initially used for the production of kojic acid. It has the following composition (g/l); glucose, 100; NH_4NO_3, 1.125; $MgSO_4.7H_2O$, 0.5; KCl, 0.1; H_3PO_4, 0.063ml.

The fungal strain was inoculated (one disk of mycelial growth, 6 days old) into 250 ml flasks, each containing 50 ml medium. The cultures were incubated static at 30ºC±2 for different time intervals. All cultures were run in duplicates. The medium was decanted, the mycelium washed several times with distilled water and dried in the oven at 80ºC for 24h. The supernatant was utilized for the determination of kojic acid, residual glucose and final pH value.

The combined medium and washings were maintained under refrigeration at 5ºC. After one night of storage, the precipitated crystals were separated by filtration. The crystals were collected, dried at 80ºC for 24h and weighed (Lin et al. 1976). For further kojic acid extraction, the filtrate was then mixed with ethyl acetate and kojic acid crystals were recovered by evaporation and weighed (Barnard and Challenger, 1949). They were combined and purified by repeated crystallization from a mixture of acetone and water (Lin et al. 1976).

Determination of kojic acid and glucose

Kojic acid was determined in culture filtrates by the method of Bentley (1957). Glucose was determined by DNS method (Chaplin, 1986).

Results and Discussion

In a previous work, among 20 fungal strains, *Aspergillus oryzae* var. *effusus* NRC 14 was found to be the most highly active organism for kojic acid production when grown on a solid glucose salts medium (Hazzaa et al., 2013). Therefore, experiments on the production of kojic acid by *A. oryzae* var. *effusus*NRC14 were carried out using a liquid glucose salts medium to follow up the course of kojic acid production and the growth of mycelium. The biochemical changes during kojic acid production are presented in (Fig. 1). Kojic acid and biomass dry weight increased as fermentation progressed to12 days (49.5 and 7.1g/l, respectively). Initial glucose was reduced from 100.0 g/l to 42.0%. Kojic acid and biomass dry weight increased as fermentation progressed up to 12 days and then decreased. As expected, the concentration of residual glucose

decreased during fermentation, coinciding with the increase in kojic acid and biomass accumulation. Such behavior of kojic acid fermentation is usually observed (May et al. 1931; Ogawa et al. 1995; Ariff et al. 1996; Saad et al. 1996; Futamura et al. 2001; Gad, 2003 and El-Aasar, 2006).

Upon testing the influence of carbon sources on the production of kojic acid as well as the growth of mycelium, different carbon sources were separately added to the medium in such amount that the final concentration (100 g/l) of carbon in the medium remained unchanged. The results in Table 1 showed that glucose was the best carbon source for kojic acid production followed by sucrose, fructose and starch (49.0, 38.0, 34.0 and 26.0 g/l, respectively), in addition to biomass dry weight yield (7.4, 6.2, 7.3 and7.5 g/l, respectively). Kojic acid was not detected when *A. oryzae* var. *effusus* NRC14 grown separately on cellulose, xylose, maltose or arabinose containing medium. The use of various carbon sources such as starch, sucrose, fructose, glucose and xylose for kojic acid fermentation by *A. oryzae* had also been investigated by Kitada et al. (1967) who suggested that kojic acid was detected in the fermentation using glucose as the carbon source, followed by sucrose and fructose, yields ranging from 0.5-0.6 g kojic acid/ g glucose could be obtained from fermentation by various kojic acid producing strains. Thus, glucose is not only used as a carbon source for biomass built- up, but it is also used as a precursor for kojic acid synthesis (Arnstein and Bentley, 1956; Kitada and Fukimbara, 1971). It is well known that glucose is the best carbon source for kojic acid production due to the similarity of its structure to that of kojic acid (Kitada et al. 1967; Basappa et al. 1970). Kitada et al. 1967; Megalla et al. 1986 suggested that, during the fermentation, kojic acid is formed directly from glucose without any cleavage of the carbon chain into smaller fragments.On the other hand, polysaccharides are a poor source of carbon for kojic acid production and very little when maltose was used (Kitada et al. 1967 and Basappa et al. 1970).

The results indicated that glucose was found to be the best carbon source for kojic acid production, accordingly glucose was tested in growth medium at different concentrations ranging from 50-150 g/l. Kojic acid production and biomass yields were recorded in Fig. 2.These results show that 10% glucose induced maximum kojic acid production and 12.5, 15.0% glucose induced maximum biomass dry weight yield (7.8, 7.8 g/l). The osmotic pressure apparently had an unfavorable effect since the production of the acid dropped off sharply, whereas, the biomass dry weight was increased. These results agreed exactly with the early results found by May et al. (1931). Kwak and Rhee, 1992 reported that, the optimum production of kojic acid was obtained at 100 g/l (10%) glucose and 0.3 g/l total amino nitrogen.

The source of nutrient nitrogen is of prime importance among the factors governing the formation of kojic acid (May et al. 1931).The effect of some inorganic nitrogen sources containing salts (0.389 N/l) on kojic acid production is shown in Table 2. Ammonium nitrate followed by ammonium di-hydrogen phosphate and ammonium mono-hydrogen phosphate were the best nitrogen sources for the production of kojic acid (49.0, 27.0 and 26.0g/l, respectively), with glucose consumption (58.0, 59.0 and 47.0g/l, respectively). On the other hand, *A. oryzea var. effusus* produced 0.84, 0.46 and 0.55 g kojic /g glucose consumed. The biomass dry weights were 7.5, 5.5 and 6.3 g/l, respectively. May et al. (1931) found that ammonium nitrate was most satisfactory.

The effect of variation in its concentration is given in Fig. 3, the results indicated that 1.125g ammonium nitrate (0.389N/l) induced maximum kojic acid production (50g/l), while 1.688g ammonium nitrate induced maximum biomass dry weight (9.0 g/l) with glucose consumed (81.6g/l). These results were in agreement with the finding of Kwak and Rhee (1992) who found that the rate of kojic acid production and glucose consumption by immobilized cell cultures increased proportionally with the increase in the nitrogen content, but at a higher nitrogen concentration there was no increase in the rate of kojic acid production, while a gradual increase in glucose consumption was observed.

The optimal pH for kojic acid production by *A. oryzae var. effusus* NRC14 appears to be 4.0 when grown on glucose as a carbon source and ammonium nitrate as the nitrogen source. The highest biomass dry weight was observed at pH 6 (Fig. 4). Most studies conducted on the effects of culture pH towards the growth and production of kojic acid was based on the initial culture pH (Lin et al. 1976; 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). On the other hand, Lin et al. (1976), showed two optimal pH values for the production of kojic acid (4.5 and 6.2) by *A. parasiticus*. Lekha and Lomane (1997) reported that enzymes, being proteins, contain ionizable groups; consequently, the pH of the culture medium effects their structure and function.

Temperature was found to have a decided effect on kojic acid production and biomass yield. Maximum kojic acid production was achieved at 30°C (49.0 g/l). The highest biomass dry weight yield was appeared at 25 °C (11.0 g/l) (Fig. 5). Kwak and Rhee, 1992 reported that the optimum temperature for kojic acid production by fungi in most of the cases was found to be 25-30°C.

The effect of medium volume (aeration) on kojic acid production and biomass dry weight yield was studied. Results in Fig. 6 showed that 50 ml of culture medium was the best volume for kojic acid production after 12 days of incubation at 30°C, while the highest yield of biomass dry weight was at 100 ml of culture medium. May et al. (1931) reported that the ratio of the surface area of the mycelium to the volume of the solution governs, to a large extent, the yields obtained, especially in shorter periods of culture. The maximum of acid were most economically produced in 12 day cultures when this ratio had a volume of from 0.3 to 0.5. Wei et al. (1991) reported that the process parameters such as pH, temperature and dissolved oxygen tension are difficult to control in the surface culture system.

Kojic acid production and biomass dry weight yield are governed by age and size of inoculum. Data presented in Tables 3 and 4 showed that one disk containing 10^6 spores of inoculum from six days of incubation was the favorable factors for kojic acid production and biomass dry weight yield. These results agreed with the results obtained by Futamura et al. (2001) who reported that, using 10^6/ml spore solution of a six days mutant *A. oryzae* MK 107-39 previously grown on PDA medium at 30°C gave a high yield of kojic acid production (28 g/ 100 g glucose/l).

Table.1 Effect of different carbohydrate sources on production of kojic acid by *Aspergillus oryzae var. effusus* NRC14

Different carbohydrate sources	Final pH	Biomass dry weight (g /l)	Kojic acid (g/l)	Kojic acid yield (%)	Residual sugar (g/l)	Consumed sugar (g/l)	g kojic acid/ g sugar consumed
Glucose	**4.44**	**7.4**	**49.0**	**49**	**41**	**59**	**0.83**
Sucrose	**3.50**	**6.2**	**38.0**	**38**	**19**	**81**	**0.47**
Fructose	**3.20**	**7.3**	**34.0**	**34**	**24**	**76**	**0.45**
Starch	**3.19**	**7.5**	**26.0**	**26**	**15**	**85**	**0.31**
Lactose	**3.26**	**0. 5**	**3.0**	**3**	**68**	**32**	**0.094**
Cellulose	**N.d**	**0.0**	**0.0**	**0.0**	**100**	**0.0**	**0.0**
Xylose	**N.d**	**0.0**	**0.0**	**0.0**	**100**	**0.0**	**0.0**
Maltose	**N.d**	**0.0**	**0.0**	**0.0**	**100**	**0.0**	**0.0**
Arabinose	**N.d**	**0.0**	**0.0**	**0.0**	**100**	**0.0**	**0.0**

Initial pH: 4,Incubation temperature: 30ºC, Incubation time: 12 day, Incubation type: Static
Kojic acid yield (%), expresses kojic acid formed of initial glucose in medium.
N.d. Means not determined.

Table.2 Effect of different inorganic nitrogen sources on production of kojic acid by *Aspergillus oryzae var. effusus* NRC14

Different nitrogen sources (Inorganic)	Final pH	Biomass dry weight (g/ l)	Kojic acid (g/l)	Kojic acid yield (%)	Residual glucose (g/l)	Consumed glucose (g/l)	g kojic acid/ g glucose consumed
NH_4NO_3	**4.60**	**7.5**	**49.0**	**49.0**	**42**	**58**	**0.84**
$NH_4H_2PO_4$	**2.32**	**5.5**	**27.0**	**27.0**	**41**	**59**	**0.46**
$(NH_4)_2HPO_4$	**2.61**	**6.3**	**26.0**	**26.0**	**53**	**47**	**0.55**
$NaNO_3$	**4.35**	**8.8**	**18.0**	**18.0**	**27**	**73**	**0.25**
$(NH_4)_2SO_4$	**2.16**	**5.0**	**13.5**	**13.5**	**34**	**66**	**0.20**
KNO_3	**4.20**	**7.3**	**12.0**	**12.0**	**38**	**62**	**0.19**
$(NH_4)_3HPO_4$	**2.80**	**5.1**	**8.0**	**8.0**	**25**	**75**	**0.11**

Table.3 Effect of different inoculum's age on production of kojic acid by *Aspergillus oryzae var. effusus* NRC14

Inoculum's age (days)	Final pH	Biomass dry weight (g/ l)	Kojic acid (g/l)	Kojic acid yield (%)	Residual glucose (g/l)	Consumed glucose (g/l)	g kojic acid/ g glucose consumed
3	3.46	6.0	36	36	38	62	0.58
6	4.31	7.4	49	49	43	57	0.86
9	4.20	6.1	39	39	48	52	0.75
12	3.30	5.8	34	34	49	51	0.67
15	3.20	5.2	28	28	52	48	0.58

Table.4 Effect of different inoculum's size on production of kojic acid by *Aspergillus oryzae var. effusus* NRC14

Inoculum's size	Final pH	Biomass dry weight (g/ l)	Kojic acid (g/l)	Kojic acid yield (%)	Residual glucose (g/l)	Consumed glucose (g/l)	g kojic acid/ g glucose consumed
0.25 of disk	3.66	4.0	36	36	38	62	0.58
0.50 of disk	3.71	5.3	39	39	31	69	0.57
1.00 of disk	4.6	7.5	50	50	41	59	0.85
1.5 of disk	3.3	7.0	44	44	45	55	0.80
2.0 of disk	3.2	6.2	33	33	36	64	0.51

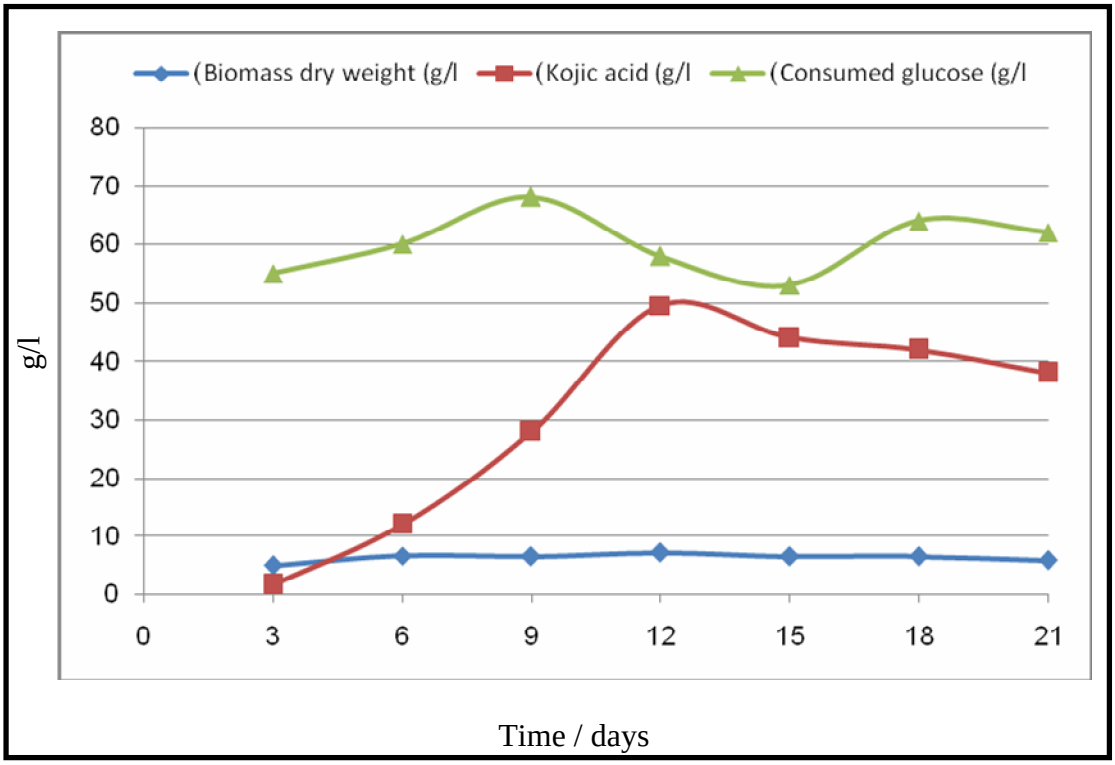

Fig.1 Effect of incubation time on production of kojic acid by *Aspergillus oryzae var.effusus* NRC14

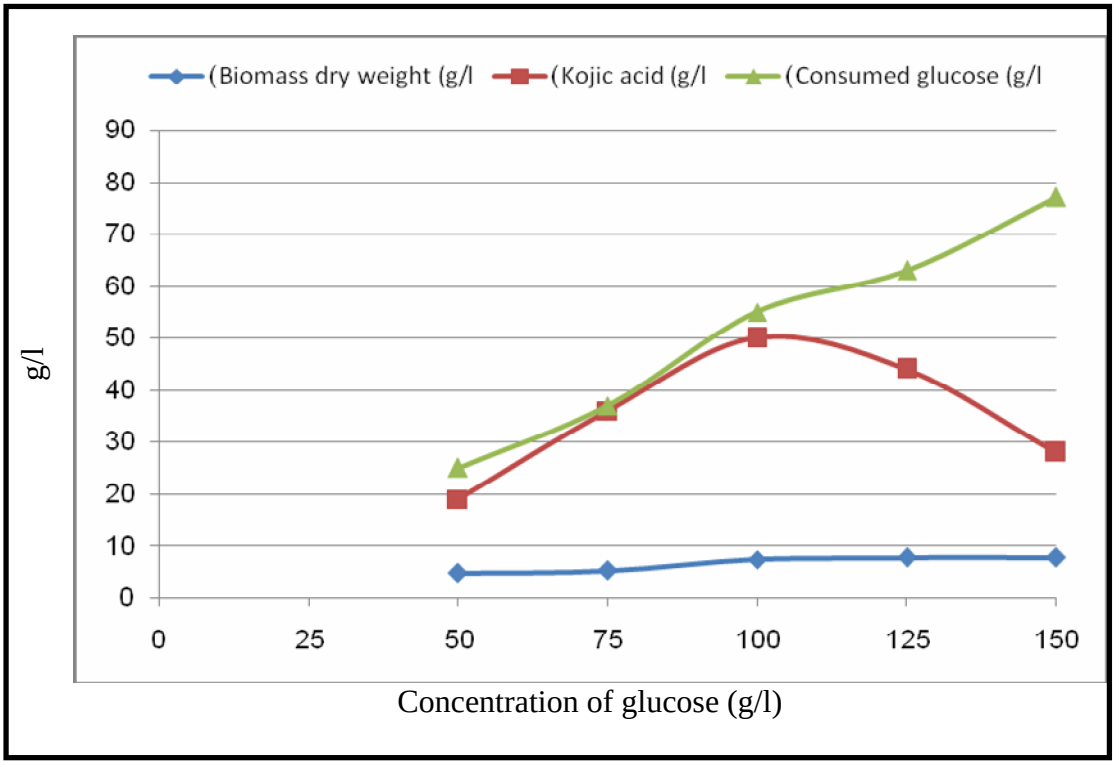

Fig.2 Effect of initial glucose concentrations on kojic acid production by *Aspergillus oryzae var.effusus* NRC14

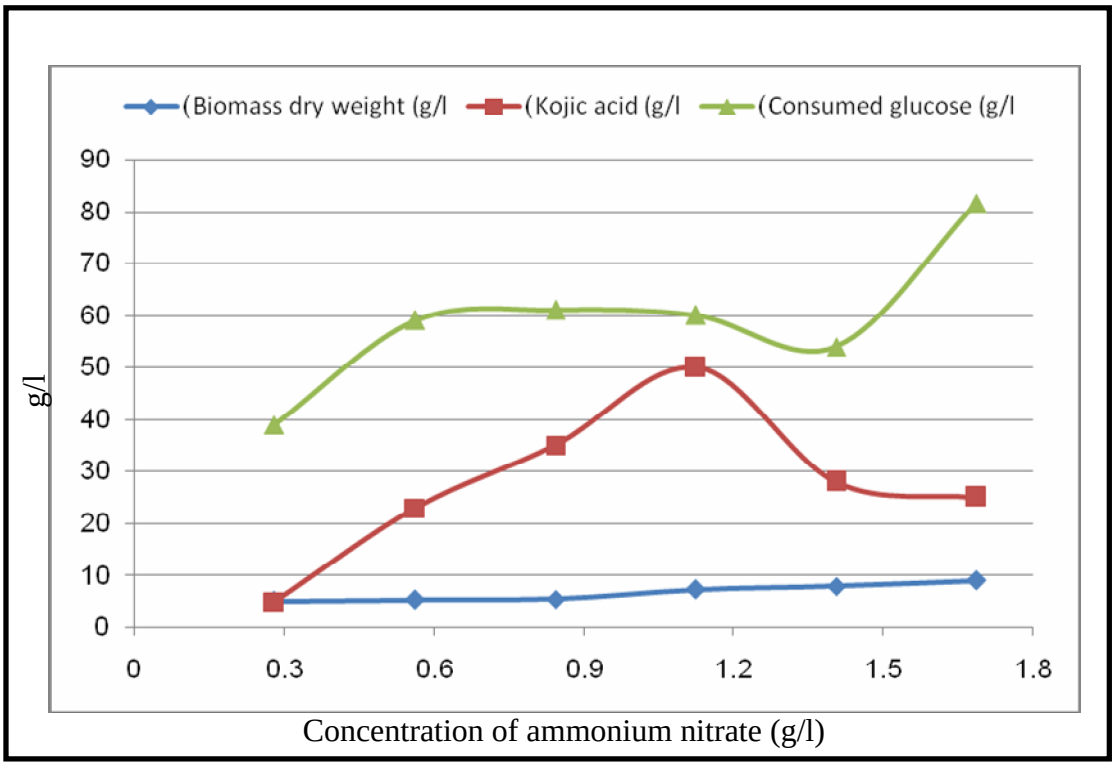

Fig.3 Effect of different concentrations of ammonium nitrate on production of kojic acid by *Aspergillus oryzae var. effusus* NRC14.

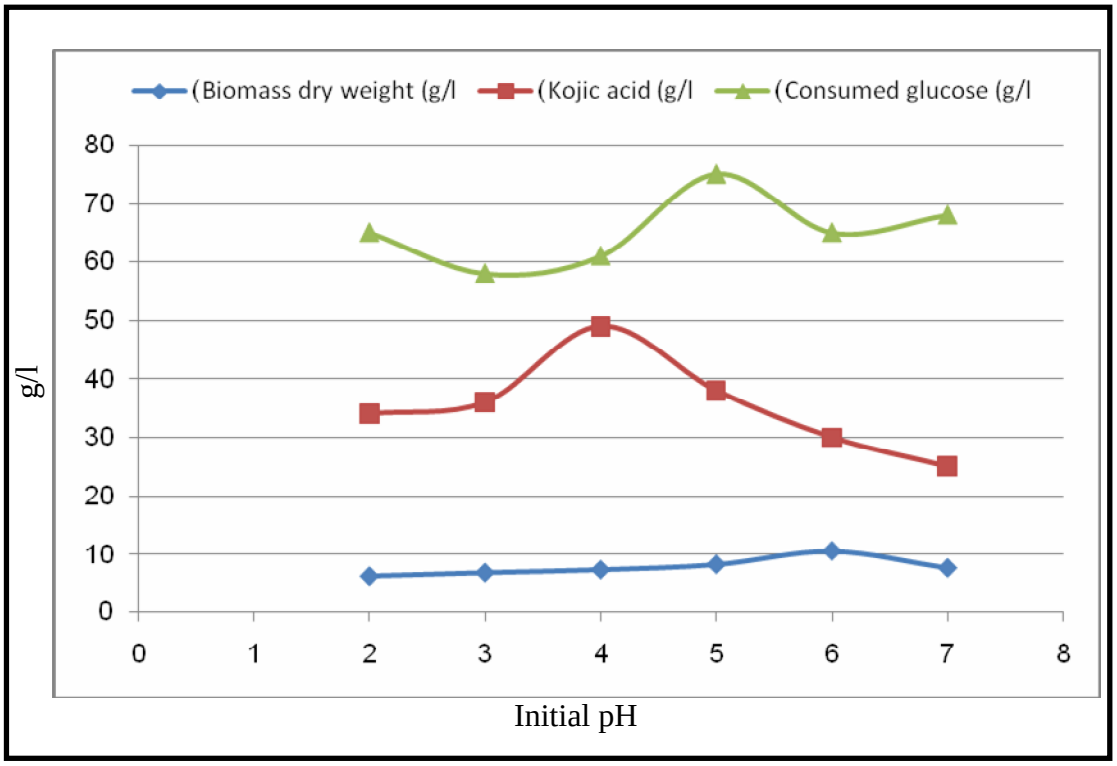

Fig.4 Effect of different pH values on production of kojic acid by *Aspergillus oryzae var. effusus* NRC14

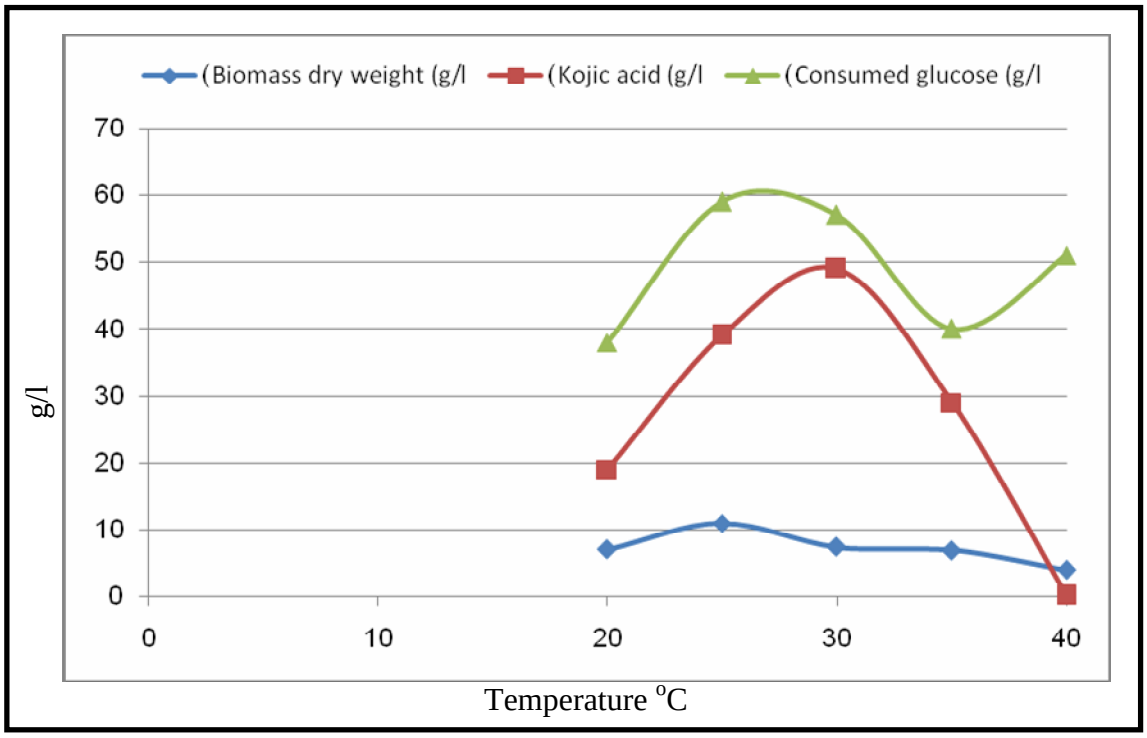

Fig.5 Effect of different temperatures (ºC) on production of kojic acid by *Aspergillus oryzae var. effusus* NRC14.

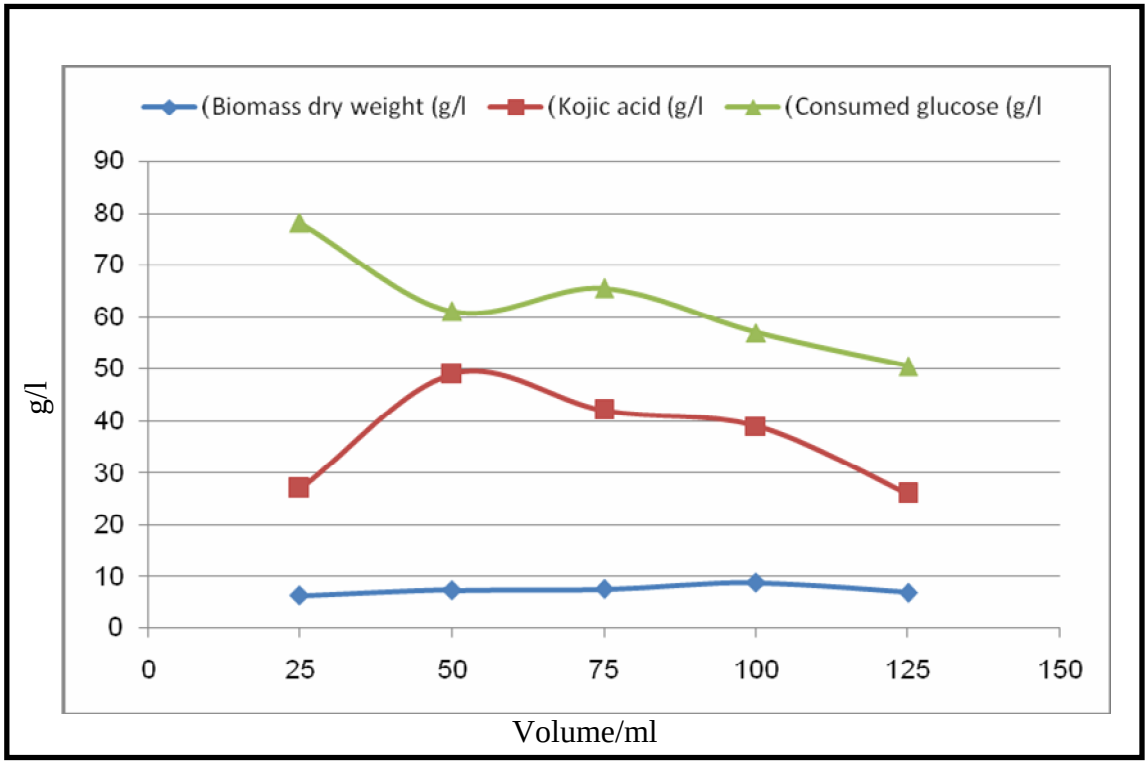

Fig.6 Effect of medium volume (aeration) on production of kojic acid by *Aspergillus oryzae var. effusus* NRC14

References

Ariff, A.B., Salleh, M.S., Ghani, B., Hassan, M.S., Rusul, G., Karim, M.I.A. 1996. Aeration and yeast extract requirements for kojic acid production by *Aspergillus flavus* Link. *Enzyme Microbiol. Technol.*, 19: 545–550.

Arnstein, H.R.V., Bentley, R. 1956. The biosynthesis of Kojic acid Production from pentose and methyl pentoses. *Biochem. J.*, 62(3): 403–411.

Barnard, D., Challenger, F. 1949. The formation of kojic acid from ethyl alcohol by *Aspergillus oryzæ*, and the action of this mould on some carbohydrate derivatives. *J. Chem. Soc.*, 1949: 110–117.

Basappa, S.C., Sreenivasamurthy, V. Parpia, H.A.B. 1970. Aflatoxin and kojic acid production by resting cells of *Aspergillus flavus* Link. *J. Gen. Microbiol.*, 61(1): 81–86.

Bentley, R. 1957. Preparation and analysis of Kojic acid. *Methods Enzymol.*, 3: 238–241.

Bentley, R. 2006. From miso, sake and shoyu to cosmetics: a century of science for kojic acid. *Nat. Prod. Rep.*, 23: 1046–1062.

Chaplin, M.F. 1986. Monosaccharides. In: Chaplin, M.F., Kennedy, J.F. (Eds.). Carbohydrate analysis: a practical approach, IRL Press, England. Pp, 1–36.

Chaudhary, J., Pathak, A.N., Lakhawat, S. 2014. Production technology and applications of Kojic acid. *Ann. Res. Rev. Biol.*, 4(21): 3165–3196.

Clevstrom, G., Ljunggren, H. 1985. Aflatoxin formation and the dual phenomenon in *Aspergillus flavus*

Link. *Mycopathol.,* 92: 129–139.

El-Aasar, S.A. 2006. Cultural conditions studies on kojic acid production by *Aspergillus parasiticus*. *Inter. J. Agri. Biol.,* 8(4): 468–473.

Futamura, T., Okabe, M., Tamura, T., Toda, K., Matsunobu, T., Park, Y.S. 2001. Improvement of production of kojic acid by a mutant strain *Aspergillus oryzae,* MK 107-39. *J. Biosci. Bioeng.,* 91(3): 272–276.

Gad, A.S. 2003. Modification of molasses for kojic acid production by *Aspergillus parasiticus*. New Egypt. *J. Microbiol.,* 5: 14–26.

Hazzaa, M.M., Saad, A.M., Hassan, H.M., Ibrahim, E. 2013. High Production of Kojic acid crystals by isolated *Aspergillus oryzae var. effusus* NRC14. *J. Appl. Sci. Res.,* 9(3): 1714–1723.

Kitada, M., Fukimbara, T. 1971. Studies on kojic acid fermentation (VII). The mechanism of the conversion of glucose to kojic acid. *J. Ferment. Technol.,* 49: 847–851.

Kitada, M., Ueyama, H., Fukimbara, T. 1967. Studies on kojic acid fermentation (I) cultural condition in submerged culture. *J. Ferment. Technol.,* 45: 1101–1107.

Kwak, M.Y., Rhee, J.S. 1992. Control mycelial growth for kojic acid production using ca-alginate immobilized fungal cells. *Appl. Microbiol. Biotechnol.,* 36: 578–583.

Lekha, P.K., Lomane, B.K. 1997. Production and application of tannin acyl hydrolase: state of the art. *Adv. Appl. Microbiol.,* 44: 215–260.

Lin, M.T., Mahajan, J.R., Dianese, J.C., Takatsu, A. 1976. High Production of Kojic acid crystals by *Aspergillus parasiticus* UNBFA12 in liquid medium. *Appl. Environ. Microbiol.,* 32(2): 298–299.

May, O.E., Moyer, A.J., Wells, P.A., Herbik, H. 1931. The production of kojic acid by *Aspergillus flavus*. *J. Am. Chem. Soc.,* 53: 774–782.

Megalla, S.E., Bennett, G.A., Ellis, J.J., Shotell, O.I. 1986. Production of deoxynivalenol and zearalenone by isolates of *Fusarium graminearum* SCHW. *J. Basic Microbiol.,* 26(7): 415–419.

Ogawa, A., Morita, Y., Tanaka, T., Sakiyama, T., Nakanishi, K. 1995. Production of kojic acid from *Aspergillus oryzae var.oryzae* by membrane-surface liquid culture. *Biotechnol. Tech.,* 9(2): 153–156.

Rosfarizan, M., Mohamed, M.S., Suhaili, N., Salleh, M.M., Ariff, A.B. 2010. Kojic acid: Applications and development of fermentation process for production. *Biotechnol. Mol. Biol. Rev.,* 5(2): 24–37.

Saad, A.M., Hamed, H., Saad, M.M. 1996. Kojic acid production by a toxigenic strain of *Aspergillus paraiticus* and a non – toxigenic strain of *Aspergillus flavus*. *Afr. J. Mycol. Biotechnol.,* 4(3): 19–27.

Wei, C.I., Huang, T.S., Fernando, S.Y., Chung, K.T. 1991. Mutagenicity studies of kojic acid. *Toxicol. Lett.,* 59: 213–220.