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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 3 (6): 977-982, 2000

Influence of pH on Kojic Acid Fermentation by *Aspergillus flavus*

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Abstract: The influence of pH on kojic acid fermentation by *Aspergillus flavus* Link 44-1 in submerged fermentation and resuspended cell system was investigated separately. Although the highest growth in submerged fermentation was obtained at initial culture pH between 6 to 7, the optimum kojic acid production was achieved at initial culture pH 3. At initial culture pH 2, growth was greatly inhibited and kojic acid was not produced. In resuspended cell system using buffered glucose solution, in which the production of cell material was carried out at initial culture pH 3, the optimum kojic acid production was also obtained at pH 3. Kojic acid production in fermentation using 50 L stirred tank fermenter with pH control strategy (fermentation was started with pH 3 and without pH control during growth phase and culture pH was controlled at 3 during the production phase) was improved by about 20% as compared to fermentation without pH control. The maximum kojic acid concentration in fermentation with pH control strategy was 62.00 g/L and this gave yield and productivity of 0.516 g/g and 0.22 G/L h, respectively.

Key words: Kojic acid, pH control strategy, *Aspergillus flavus*, resuspended cell system

Introduction

Kojic acid is used in medical field as painkiller and an anti-inflammation drug (Anonymous, 1992). In food industry, kojic acid is used as a precursor for flavor enhancers (Le Blanch and Akers, 1989) 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).

Medium pH is one of the important parameters affecting growth and product formation during any fermentation process. Kojic acid can be produced by several fungi such as *A. oryzae* and *A. flavus* at a wide pH range (3 to 7). Substantially high kojic acid production by *A. flavus* in submerged fermentation was obtained at pH between 6 to 7 (Basappa *et al.,* 1970) and also at pH 3 (Madihah *et al.,* 1993). Kojic acid can also be produced by *A. flavus* in resuspended cell system using buffered glucose solution at pH 6.5, in which cells were first produced using yeast extract-sucrose (YES) medium. Good performance of kojic acid production was also obtained by immobilized cell of *A. oryzae* at pH 3 (Kwak and Rhee, 1992).

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 were produced and kojic acid is synthesized by the direct conversion from 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 resuspended in buffered glucose solution (Ariff *et al*., 1996; Bajpai *et al.,* 1982). There must be an optimum culture pH for growth of kojic acid-producing fungus and enhancement of secretion of the enzymes responsible for kojic acid synthesis and also an optimum solution pH for the activities and stability of these enzymes. However, reports on these are still not available in the literature.

In this study, the effect of pH on kojic acid fermentation by *A. flavus* Link 44-1 in submerged fermentation and resuspended cell system was investigated separately. The results obtained were used to develop the culture pH control strategy aimed at optimizing the batch production of kojic acid in a 50 L fermenter.

Materials and Methods

Microorganism and Medium: The fungus, *Aspergillus flavus* Link 44-1 was used for kojic acid production. The inoculum was prepared according to the method as described previously (Ariff *et al.,* 1997). A standard inoculum of 3.5×10^4 spores/mL medium was used in all experiments. The medium for kojic acid production consisted of (in g/L): glucose, 100; yeast extract, 5; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.5 and methanol (10 mL/L).

Submerged Fermentation and Resuspended Cell System in Shake Flasks: Submerged fermentation of kojic acid was carried out in a shake flask. The 250 mL flasks containing 150 mL medium with different initial pHs (2, 3, 4, 5, 6 and 7) were inoculated with spore suspension. The flasks were incubated at 30°C on a rotary shaker and agitated at 250 rev/min.

Resuspended cell system was carried out as follows. The production of cell material was first carried out in a shake flask using the same medium and cultural conditions as in submerged fermentation. After growth reached a stationary phase (96 h), cells were harvested by centrifuging at 10,000 rev/min for 20 min at 4°C. The cell pellets were washed with sterile deionized water and recentrifuged. Small volume of salt solution was added to the cell pellets to produce a concentrated cell suspension. The same volumes of concentrated cell suspension were then resuspended into shake flasks containing sterile 150 mL of 50 g/L glucose at different pHs in HCl-KCl buffer (pH 2), citrate-phosphate buffer (pH 3 to 5) and phosphate buffer (pH 6 to 7). The flasks were incubated at 30°C on rotary shaker agitated at 150 rev/min. All experiments were carried out in triplicate and data presented are means values.

Fermentation in a 50 L Stirred Tank Fermenter: A 50 L stirred tank fermenter (Biostat U, B. Braun, Melsungen, Germany) was used in this study. Three six-bladed turbine impellers with a diameter (D) of 125 mm mounted on the agitator shaft were used for agitation. The fermenter was equipped with temperature, dissolved oxygen and pH controllers. During the fermentation, agitation speed (N) was fixed at 250 rev/min (impeller tip speed = πND = 3.27 m/s) and the dissolved oxygen tension (DOT) in the culture broth was controlled via a sequential cascade control of airflow rate. The maximum and minimum set points of permitted airflow rates were 37.5 L/min and 2.5 L/min, respectively. The polarographic dissolved oxygen probe (Ingold, Switzerland) was used to measure DOT levels. During the fermentation, DOT level and culture pH were recorded continuously by using a recorder (Kipp and Zonen, Holland)

connected to the main controller. The initial pH of the culture was adjusted to 3 and the culture pH was controlled at pH 3 during the production phase by the automatic addition of either 0.2 N HCl or 0.2 N NaOH. The optimum aeration control strategy for maximum batch production of kojic acid was employed (Ariff *et al*., 1996). In this aeration control strategy, DOT during active growth phase was controlled at 80% saturation and then switched to 30% saturation during the production phase. The temperature within the fermenter was maintained at 30°C.

Analytical Determinations: During the fermentation and reaction, samples were withdrawn at various time intervals for analysis. Samples were filtered using preweighed microfiber filter. The supernatants were used for kojic acid and glucose determination while the residues were dried in an oven at 85°C for dry cell weight measurement. Kojic acid was determined by using high Performance Liquid Chromatography (HPLC) method with UV detector at 265 nm as described previously (Ariff *et al.,* 1997). Glucose was determined enzymatically by glucose oxidase using Sigma Diagnostics Glucose (Trinder) reagent (Catalog number 315-100) and the absorbance was measured at 505 nm.

Enzyme Assays: The procedure for preparation of homogenate for enzymes assay was estimated according to Bajpai *et al*. (1981) and the assay methods for each enzyme are as follows; Hexokinase (EC 2.7.1.1) was assayed in 0.1 M glycylglycine buffer, pH 9.0 (Darrow and Colowick, 1962), 6-phosphogluconate dehydrogenase (EC 1.1.1.44) in 0.1 M triethanolamina/HCl buffer, pH 7.6 (Pontremoli and Grazi, 1966), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) in 0.1 M glycylglycine buffer, pH 8.0 (Kuby and Noltmann, 1966), glucose dihydrogenase (EC 1.1.1.47) in 0.1 M phosphate buffer, pH 6.0 (Hauge, 1966), gluconate dehydrogenase (EC 1.1.99.3) in 0.5 M sodium acetate buffer, pH 5.5 (Wood *et al*., 1962). One unit of enzyme activity was defined as the amount of enzyme, which catalysed the conversion of 1 µmol substrate into product min^{-1}. Specific activity was expressed in terms of units $(mg\ protein)^{-1}$. Protein was estimated by the Lowry method using bovin serum albumin as reference (Eggstein and Kreuz, 1967).

Results and Discussion

Effect of Initial pH on Kojic Acid Production in Submerged Fermentation: The time courses of kojic acid fermentation by *A. flavus* at different initial culture pHs are shown in Fig. 1 (A-D) and the performance of each fermentation is given in Table 1. Growth increased with increasing initial culture pH up to 5 and no lag phase was observed except at initial culture pH 2. However, growth at an initial culture pH between 5 to 7 was not significantly different and growth reached a stationary phase after about 144 h with maximum cell concentration of around 18.00 g/L. At initial culture pH 3 and 4, a stationary phase was achieved after about 216 h and 288 h with maximum cell concentration of 14.70 g/L and 16.40 g/L, respectively. A long lag phase (144 h) and very slow growth was observed at initial pH 2, suggesting that growth of *A. flavus* was greatly inhibited at culture pH 2.

Different initial culture pHs greatly influenced kojic acid production by *A. flavus* (Fig. 1B). At initial culture pH ranged between 4 to 7, kojic acid production stopped after about 216 h and the maximum concentration obtained was between 2.50 g/L to 19.20 g/L. On the other hand, at initial culture pH 3, rapid kojic acid production continued up to 360 h. Maximum cell concentration obtained during the fermentation increased with increasing initial culture pH. However, the highest kojic acid production (30.20 g/L) was obtained at initial culture pH 3. In order to investigate whether the fungi produced the enzyme effectively or if production is simply proportional to cell mass, the maximum amount of kojic acid produced was determined per unit cell weight (P_{max}/X_{max}). The value of P_{max}/X_{max} was the highest (2.05 g/g) at pH 3 and sharply decreased to values of between 1.17 g/g to 0.14 g/g at initial culture pH 4 and above (Table 1). Although high initial culture pH (>4) enhanced growth of *A. flavus*, the ability of the cells to convert glucose to kojic acid was very low. Because growth was very low, kojic acid was not produced in fermentation with initial culture pH 2.

Glucose consumption increased with increasing culture pH and this was paralleled with growth (Fig. 1D). At initial culture pH 4 to 7, pH was decreased sharply during the early stages and then increased towards the end of the fermentation (Fig. 1C). Reduction in culture pH may possibly due to the accumulation of kojic acid and other organic acids. Beside kojic acid, other organic acids such as oxalic, succinic, lactic, acetic and citric acid were also produced during the fermentation (Table 2). Substantially high accumulation of oxalic acid was observed at initial pH 4 to 7. The highest production of succinic acid was occurred at the same initial pH for kojic acid production. Reduction in oxalic acid production at initial pH 3 may be one of the possible reasons for an increase in kojic acid production. It is important to note that excessive reduction of kojic acid and increment in production of oxalic acid, succinic acid and lactic acid were observed towards the end of fermentations with initial culture pHs 5 to 7. Under glucose depleted conditions, mycelium of kojic acid producers degraded kojic acid to other compounds such as oxalic and acetic acids (Bajpai *et al*., 1981; Clevstrom and Liunggren, 1985).

From the results of this study, it can be concluded that the lower kojic acid production at initial culture pH $\geq$5 may be due to; (i) The culture pH was not suitable for the enhancement of the secretion and also not optimum for the activities and stability of the enzymes involved in kojic acid metabolic pathway, (ii) The culture pH caused excessive degradation of kojic acid to other compounds and (iii) Most of glucose supplied was used for abundant cell growth and the amount remained in the culture towards the end of the fermentation for a conversion to kojic acid was very little. However, the results obtained from this experiment cannot be used to explain the actual caused of lower production at initial culture pH>5. In order to investigate the real effect of pH on the above matter, subsequent experiment of kojic acid production using resuspended mycelial system at different pHs was also carried out.

Effect of pH on Kojic Acid Production in Resuspended Cell System: Fig. 2 shows kojic acid production in resuspended cell system at different pHs, in which the cell materials were produced from culture at initial pH 3. Cell concentration remained unchanged during the incubation, indicating that growth did not occur possibly due to unavailability of nitrogen source in the culture medium (data not shown). This means that glucose was consumed either for conversion to kojic acid or other unknown substances. Kojic acid production and glucose consumption were greatly influenced by the pH. At pH 2 to 4, kojic acid production increased while glucose concentration decreased almost linearly with incubation time. The highest kojic acid production (12.50 g/L) was obtained at pH 3 followed by pH 2 and 4. Very low kojic acid production was obtained at pH ranging from 5 to 7, though glucose was consumed rapidly during the incubation. These results suggest that pH ranging from 2 to 4 were favorable for the activities and stability of enzymes relevant to kojic acid synthesis.

The effect of initial culture pH on the efficiency of the cell materials for subsequent used in kojic acid production using resuspended cell system at pH 3 is shown in Fig. 3 (A-B). The

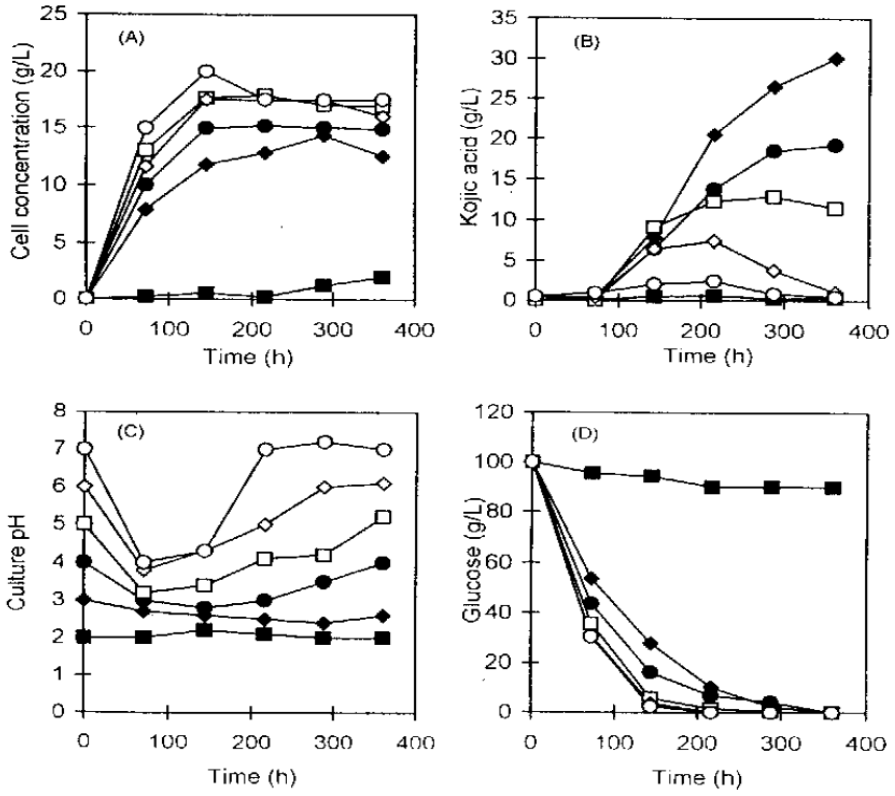

Fig. 1: Effect of initial pH on kojic acid production by *A. flavus* in batch submerged fermentation. (A) Cell concentration, (B) Kojic acid, (C) pH and (D) Glucose. (■) pH 2; (♦) pH 3; (●) pH 4; (□) pH 5; (◊) pH 6 and (O) pH 7

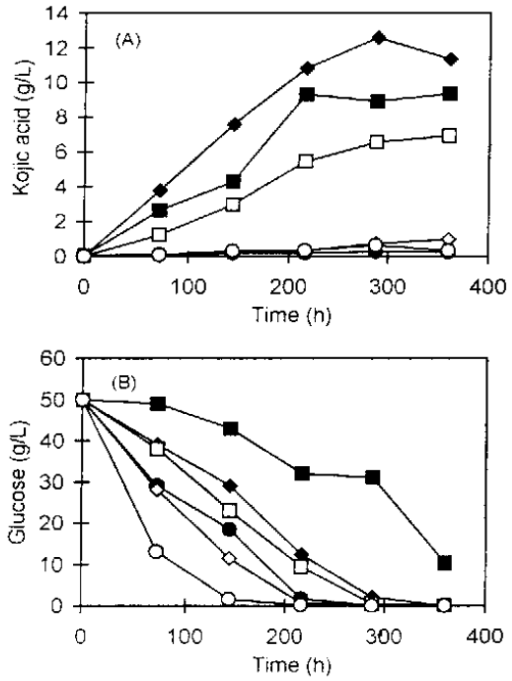

Fig. 2: Effect of pH on kojic acid production in resuspended cell system. A. flavus biomass was produced in cultivation with an initial culture pH of 3. (A) kojic acid and (B) glucose (■) pH 2; (♦) pH 3; (●) pH 4; (□) pH 5; (◊) pH 6 and (O) pH 7

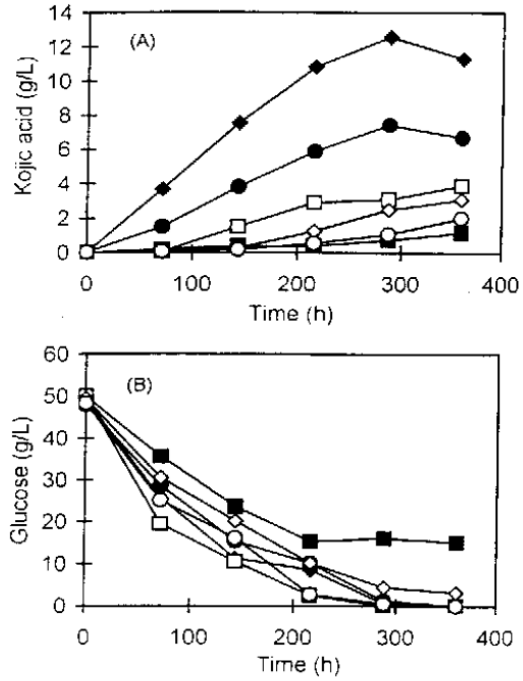

Fig. 3: Effect of initial culture pH on the production of *A. flavus* biomass for subsequent used in resuspended mycelial system of kojic acid production at pH of 3. (A) Kojic acid and (B) Glucose.(■) pH 2; (♦) pH 3; (●) pH 4; (□) pH 5; (◊) pH 6 and (O) pH 7

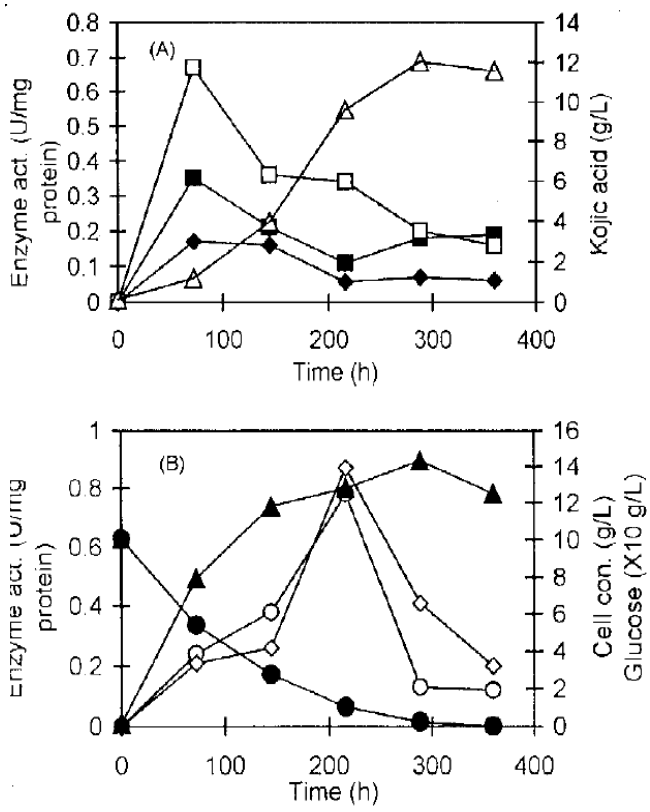

Fig. 4: Enzyme activities in *A. flavus* mycelium grown in batch submerged culture. Symbols represent, (A): (■) glucose dehydrogenase; (✦) gluconic dehydrogenase; (□) glucose-6-phosphate dehydrogenase; (△) kojic acid and (B): (O) hexokinase; (◇) 6-phosphogluconate dehydrogenase; (●) glucose and (▲) cell concentration

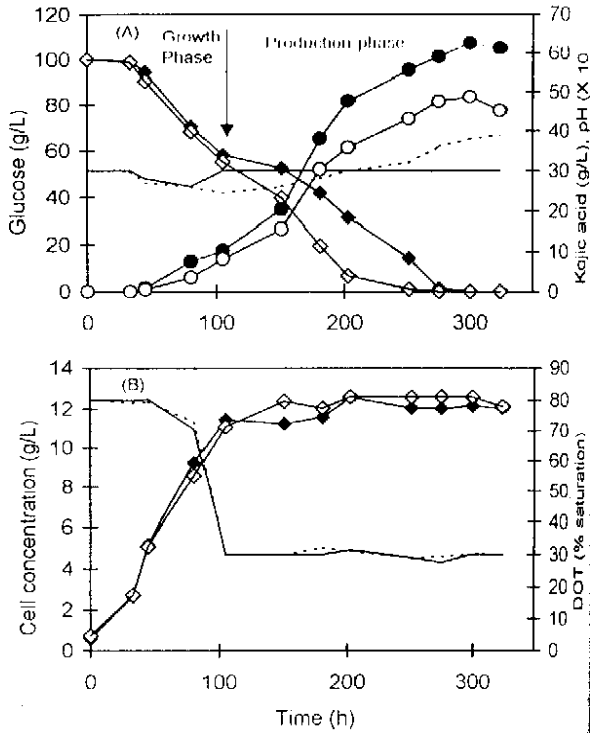

Fig. 5: Comparison of kojic acid production in batch fermentation without and with pH control strategy in 50 L stirred tank fermenter. (A); (■) Kojic acid; (✦) Glucose and (___) pH in fermentation without pH control and open symbols represent kojic acid, glucose and pH profile, respectively, in fermentation with pH control. (B); (●) Cell concentration and (___) DOT in fermentation without pH control and open symbols represent cell concentration and DOT level in fermentation with control of pH

efficiency of cell materials in synthesizing kojic acid, which was depended upon the activities of cell-bound enzymes relevant to kojic acid synthesis, was greatly influenced by the initial culture pH. At a range of the initial culture pH (2-7) investigated, the highest kojic acid production in resuspended cell system was obtained when cell materials from culture at initial pH 3 was used followed by initial culture pH 4 and 5. Very low kojic acid production was observed when cell materials from initial culture pH 2 and 7 were used. These results indicate that initial culture pH of between 3 to 4 were favorable for the production of cell materials which have high efficiency in synthesizing kojic acid.

Enzymes involved in kojic acid production: During growth of *A. flavus* in batch submerged fermentation using shake flasks culture at initial pH 3, the specific activities of glucose dehydrogenase, gluconic dehydrogenase and glucose-6-phosphate dehydrogenase reached a maximum after 72 h, i.e. during the active growth phase (Fig. 4A). On the other hand, hexokinase and 6-phosphogluconate-dehydrogenase increased concomitantly with growth and became maximum at a stationary growth phase (Fig. 4B). Rapid kojic acid production was observed when the activities of hexokinase and 6-phosphogluconate dehydrogenase were at maximum. All these enzymes have been reported for their possible participation in kojic acid synthesis (Bajpai *et al*., 1981; Nandan and Polasa, 1985).

The secretion of these enzymes during growth phase was closely related with the formation of intermediates for further used in the synthesis of kojic acid during production phase. Gluconic acid-δ-lactone and at least one of the three compounds, 3-ketogluconic acid lactone, 3-ketoglucose and oxykojic acid, are believed to be intermediates in the hypothetical kojic acid metabolic pathway (Bajpai *et al*., 1981). Glucose dehydrogenase is required to convert glucose to D-glucose-δ-lactone, which is then converted to 3-ketogluconic-acid-lactone by gluconate dehydrogenase. The specific activities of all the six enzymes were slightly decreased as the fermentation progressed. Although relatively high activities of these enzymes were maintained toward the end of the fermentation, kojic acid production ceased when glucose was depleted. During the fermentation, glucose was directly converted to kojic acid through a multi-steps enzyme reaction (Arnstein and Bentley, 1953). A possible approach to increase the production might be the extension of the fermentation process by adding glucose intermittently to the culture, which still contained very active cell-bound enzymes. Substantially high activities of all the six enzymes were also detected in resuspended cell system at pH 2 to 4 (data not shown).

Table 1: Comparison of the performance of kojic acid fermentation by *A. flavus* carried out at different initial culture pH

Initial culture pH	X_{max} (g/L)	P_{max} (g/L)	P_{max}/X_{max} (g/g)	$Y_{p/s}$ (g/g)
2	2.30	0.60	0.26	0.06
3	14.70	30.20	2.05	0.30
4	16.40	19.20	1.17	0.19
5	18.90	12.90	0.68	0.13
6	17.80	7.50	0.42	0.07
7	17.20	2.40	0.14	0.02

Maximum cell concentration, X_{max}; maximum kojic acid concentration, P_{max} and yield of kojic acid based on glucose consumed, $Y_{p/s}$

Table 2: Kojic acid and several organic acids production in batch submerged fermentation of *A. flavus* at 216 h and 360 h of fermentation

Organic acids (g/L)	Initial culture pH					
	2	3	4	5	6	7
At 216 h of fermentation						
Kojic acid	0.00	21.56	13.21	11.79	7.12	2.83
Oxalic acid	0.57	6.99	0.50	9.91	0.96	0.08
Succinic acid	0.45	2.17	0.18	0.06	0.06	0.08
Lactic acid	0.13	0.80	0.01	0.18	0.59	0.14
Acetic acid	0.05	0.17	2.89	0.02	0.04	0.02
Citric acid	0.13	0.71	0.01	0.01	0.03	0.03
At 360 h of fermentation						
Kojic acid	0.01	30.20	19.20	10.23	2.23	0.16
Oxalic acid	0.38	0.15	3.16	3.96	3.52	3.40
Succinic acid	0.71	7.70	0.50	0.31	0.91	0.37
Lactic acid	0.20	0.61	0.46	0.45	1.24	0.60
Acetic acid	0.07	0.07	0.14	0.02	0.02	0.03
Citric acid	0.20	0.22	0.05	0.02	0.03	0.03

Table 3: Comparison of the performance of kojic acid fermentation by *A. flavus* in a 50 L fermenter without and with pH control.

Parameters	Without pH control	pH control strategy
X_{max} (g/L)	12.56	12.01
P_{max} (g/L)	49.00	62.00
P_{max}/X_{max} (g/g)	3.90	5.16
t_f (h)	240.00	280.00
Productivity (g/L.h)	0.20	0.22

Fermentation time, t_f

pH control strategy in 50 L fermenter: The preceeding results indicates that appropriate pH control strategy may be applied for improvement of kojic acid production. Based on this, batch fermentation in 50 L fermenter with pH control strategy (fermentation was started with initial culture pH 3, pH was not controlled during growth phase and pH was controlled at 3 during production phase) was performed. In comparison, fermentation started with initial pH 3 and was not controlled throughout the fermentation. Fig. 5 shows time course for fermentation with and without pH control strategies in 50 L fermenter. The performance of the fermentation is summarized in Table 3. Growth was not significantly different with different pH control strategies. Glucose consumption rate during growth phase was also not significantly different for both fermentation but the rate during production phase was higher for fermentation with pH control strategy than without pH control. Kojic acid production was improved by about 20% higher in fermentation with pH control strategy (62.00 g/L) as compared to fermentation without pH control (49.00 g/L), though the time (280 h) to reach maximum concentration was about the same. The P_{max}/X_{max} value for fermentation with pH control strategy (5.16 g/g) was significantly higher than for fermentation without pH control (3.90 g/g), indicating that the cell-bound enzymes in fermentation with pH control strategy acted effectively for kojic acid synthesis than the latter.

In both fermentations, culture pH was decreased to around 2.0 after about 90 h. For fermentation without pH control, culture pH dropped to below 2 during the early stages of production phase and then increased to pH 4 towards the end of fermentation. For fermentation with pH control strategy, culture pH was successfully maintained at pH 3 throughout the production phase. It is interesting to note that a slight change in culture pH during the production phase greatly affected kojic acid synthesis. This result indicates that the activity and stability of the enzymes that were responsible for kojic acid were enhanced when the culture pH during the production phase was maintained at 3. In other words, the activity and stability of the cell-bound enzymes relevant to kojic acid synthesis are very sensitive to a slight change in pH and optimal at pH of around 3. This is supported by the observation that glucose consumption rate for conversion to kojic acid was higher in fermentation with pH control than fermentation without pH control.

From this study, it was found that the culture pH greatly influenced kojic acid production in submerged fermentation and resuspended cell system. Several enzymes such as glucose dehydrogenase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and hexokinase were found relevant to kojic acid synthesis by *A. flavus*. Fermentation with initial culture pH 3 and pH was not controlled during growth phase was favorable for production of cell materials, which contained high activities of enzymes relevant to kojic acid synthesis. The optimum pH for activities and stability of these cell-bound enzymes was 3. Kojic acid production in batch submerged fermentation can be improved by starting the

fermentation at initial culture pH 3 and then culture pH was only controlled constant at 3 after growth reached a stationary phase or during the production phase.

Acknowledgement

The authors are indebted to Universiti Putra Malaysia (UPM) for the research facilities and the Ministry of Science, Technology and Environment under Intensification Research in Priority Areas (IRPA) Research Program for the funding.

References

Anonymous, 1992. Personal communications. Sansei Pharmaceutical Company, Japan.

Ariff, A.B., M. Rosfarizan, L.S. Herng, S. Madihah and M.I.A. Karim, 1997. Kinetics and modelling of kojic acid production by *Aspergillus flavus* link in batch fermentation and resuspended mycelial system. World J. Microbiol. Biotechnol., 13: 195-201.

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

Arnstein, H.R.V. and R. Bentley, 1953. The biosynthesis of kojic acid. 1. Production from [1-^{14}C] and [3:4-$^{14}C_2$] glucose and [2-^{14}C]-1:3-dihydroxyacetone. Biochem. J., 54: 493-508.

Bajpai, P., P.K. Agrawala and L. Vishwanathan, 1981. Enzymes relevant to kojic acid biosynthesis in *Aspergillus flavus*. Microbiology, 127: 131-136.

Bajpai, P., P.K. Agrawala and L. Vishwanathan, 1982. Production of kojic acid by resuspended mycelia of *Aspergillus flavus*. Can. J. Microbiol., 28: 1340-1346.

Basappa, S.C., V. Sreenivasamurthy and H.A. Parpia, 1970. Aflatoxin and kojic acid production by resting cells of *Aspergillus flavus* link. Microbiology, 61: 81-86.

Chen, J.S., C. Wei and M.R. Marshall, 1991. Inhibition mechanism of kojic acid on polyphenol oxidase. J. Agric. Food Chem., 39: 1897-1901.

Clevstrom, G. and H. Liunggren, 1985. Aflatoxin formation and the dual phenomenon in *Aspergillus flavus* link. Mycopathologia, 92: 129-139.

Darrow, R.A. and S.P. Colowick, 1962. Hexokinase from Baker's yeast: ATP + Hexose→ADP + Hexose-6-phosphate + H+. Methods Enzymol., 5: 226-235.

Eggstein, M. and F.H. Kreuz, 1967. Techniques in Protein. In: Biochemistry of Food Proteins, Hudson, B.J.F. (Ed.). Elsevier Applied Science, UK., pp: 307-351.

Hauge, J.G., 1966. Glucose dehydrogenases-soluble: II. *Bacterium anitratum*. Methods Enzymol., 9: 107-111.

Kuby, S.A. and E.A. Noltmann, 1966. Glucose 6-phosphate dehydrogenase (crystalline) from brewer's yeast. Methods Enzymol., 9: 116-125.

Kwak, M.Y. and J.S. Rhee, 1992. Cultivation characteristics of immobilized *Aspergillus oryzae* for kojic acid production. Biotechnol. Bioeng., 39: 903-906.

Le Blanch, D.T. and H.A. Akers, 1989. Maltol and ethyl maltol from larch tree to successful food additives. Food Technol., 26: 78-87.

Madihah, M.S., A.G. Baharuddin, M.I.A. Karim, M.A. Hassan and K. Mitsugi *et al*., 1993. Cultivation characteristics during kojic acid fermentation by local fungus, mutant strain 44-1. Proceedings of the 2nd UNESCO National Workshop on Promotion of Microbiology in Malaysia, January 28-29, 1991, Serdang, Selangor, Malaysia.

Nandan, R. and H. Polasa, 1985. Inhibition of growth of kojic acid biosynthesis in *Aspergillus* by some chlorinated hydrocarbons. Indian J. Microbiol., 25: 21-25.

Ohyama, Y. and Y. Mishima, 1990. Melanosis-inhibitory effect of kojic acid and its action mechanism. Fragrance J., 6: 53-58.

Pontremoli, S. and E. Grazi, 1966. 6-Phosphogluconate dehydrogenase-crystalline. Methods Enzymol., 9: 137-141.

Wood, W.A., R.A. Fetting and B.C. Hertlein, 1962. Gluconic dehydrogenase from *Pseudomonas fluorescens*: Gluconate + 2 ferricyanide→2-Ketogluconate + 2 ferrocyabide. Methods Enzymol., 5: 287-291.