Development of process control for improvement of kojic acid fermentation process by *Aspergillus* spp.

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Key words: kojic acid, process control, *Aspergillus* spp, pH and DOT control strategy, scaling up

Introduction

Kojic acid, 2-hydroxymethyl-5-hydroxy-γ-pyrone, is an organic acid that has many commercial applications such as an anti-inflammatory drug and painkiller (Kayahara et al., 1990), a precursor for flavour enhancers and as an antibrowning agent, which inhibits polyphenol oxidase, an enzyme responsible for blackening of agricultural products (Le Blanch and Akers, 1989). It is also used as a preservative and also as antioxidant for oils and fats (Hein, 1999) as well as used as a whitening agent and a protective against ultraviolet light in cosmetic (Ohyama and Mishima, 1990). It is widely used in combination with alpha-hydroxy acid in the formulation of skin whiteners or whitening products that can be used to control melanin production, lighten freckles and age spot. The use of kojic acid to replace hydroquinone that bleaches and possibly damage skin in cosmetic products is increasing enormously. Kojic acid is recognised as important intermediates in the production of chemicals that can be used as pharmaceutical. Kojic acid is present naturally in Koji malt (*Aspergillus oryzae*). Koji malt has been used for production of many traditional Japanese foods such as soybean paste (miso), soybean sauce (shoyu), and Japanese wine (sake) for a long time in Japan. In Japan, kojic acid is believed to have a capability to improve health and beauty, and health foods containing kojic acid are widely marketed. The application of kojic acid is increasing enormously with a growing presence in the industry related to its application. At present, there is a strong demand for this compound and only a few companies around the world such as Tokyo Kasei Kogyo Co., Ltd., Japan, Shanghai Greenmen International Trading Co., Ltd., China and Jarchem Industries Inc., USA have capability to produce kojic acid for commercial use. The current market price of kojic acid is quite high. It is ranging from RM 950.00 - 5000.00 per kg for industrial grade and RM 11 000.00 – 19 000.00 per kg for the laboratory grade. It is expected that the market of kojic acid will increase throughout the year. Kojic acid is produced industrially by aerobic fermentation of *Aspergillus* spp. Although it has been produced and applied industrially for some time, the subject is still extensively studied. Publications on the production of kojic acid by means of fungal fermentation are limited and heavily protected by patents. In addition, details of industrial techniques of fermentation are rarely revealed since they are know-how of each producing company. Thus, an attempt to produce the compound at large scale is restricted. The scope of this study was focussed on development of process aimed at establishing high performance kojic acid fermentation using local isolate, *Aspergillus flavus* strain 44-1.

Materials and Methods

The fungus, *Aspergillus flavus* Link strain 44-1 was used for kojic acid production. The experiment in the fourth stage was focussed on the identification of the kojic acid fermentation characteristics of *A. flavus* in batch cultures, such as the specific growth rate, glucose consumption rate, yield and productivity using different concentrations of sucrose and fructose as carbon sources. The biotransformation process of sugars (glucose, sucrose and fructose) to kojic acid by cell-bound enzymes of *A. flavus* in shake flask was carried out in the fifth stage. The effect of cell concentration, pH and carbon source concentration on the transformation rate was investigated. The mathematical models proposed for the biotransformation process or resuspended cell system were then tested on the experimental data gathered in this stage. In the final stage, batch kojic acid fermentations using glucose and gelatinized sago starch as carbon sources were carried out in 50 L stirred tank fermenter. In this experiment, some of the important fermenter operating variables such as impeller tip speed and air flow rate; and control strategy such as pH and DOT that were important in the scaling-up of the process, was identified. Three different sizes of stirred tank fermenter (2 L, 8 L and 50 L) were used throughout this study. All the fermenters were equipped with pH, temperature and dissolved oxygen tension (DOT) control systems. The temperature within the fermenter vessel was controlled by means of the external double wall or thermostat jacket. The control system for pH and DOT for each fermenter is described in more details in the chapter where they were used in the experiment. During the fermentation excessive foaming was occurred, especially during active growth phase. In all fermentations, foam was controlled manually by adding few drops of an antifoam (silicone based antifoam, SIGMA) at time intervals during growth phase only. Since the problem of foaming was not very serious and the antifoam used was effective, only very little antifoam (less than 0.1% v/v) was added throughout each fermentation.

Results and Discussion

Improvement of kojic acid (5-hydroxy-2-hydroxymethyl-δ-pyrone) fermentation by a local strain, *Aspergillus flavus* Link 44-1, could be achieved either through strain improvement approach or process development approach. In our study, kojic acid fermentation by *A. flavus* in the 2 L fermenter was further improved by using optimal pH control strategy, which produced comparable kojic acid concentration as obtained in shake flask culture. In comparison to fermentation without pH control, improvement of 123% was achieved, in term of kojic acid concentration. In term of overall productivity, higher percentage of improvement was achieved because the fermentation time was greatly reduced. From our study, besides DOT and pH control strategies, it was also found that the impeller tip speed was one of the important parameters in scaling-up of the fermentation from 2 L to 50 L stirred tank fermenter. Using the optimal impeller tip speed, kojic acid production in 50 L fermenter was improved by about 380% as compared to fermentation with non-optimal impeller tip speed. Scale-up based on a constant impeller tip speed could be used to obtain kojic acid production in 50 L fermenter (62.5 g/L), and this was significantly higher than that obtain in 2 L fermenter (36.5 g/L). The yield and overall productivity obtained in 50 L fermenter were 0.62 g kojic acid/g glucose and 0.223 g/L.h, respectively. These values are significantly higher than those reported in the literature for batch submerged fermentation (Kitada et al., 1967; Takamizawa et al., 1998; Ariff et al., 1996; Ogawa et al., 1995), which was in the range of 0.24-0.51 g kojic acid/g glucose and 0.13 - 0.185 g/L.h for yield and overall productivity, respectively.

A conventional batch process could be used for high production of kojic acid using glucose, starch hydrolysate and sucrose. Nevertheless, the optimal aeration control strategy (two-phase DOT control) is a must for kojic acid fermentation in stirred tank fermenter for all types of carbon source used. Although kojic acid fermentation by *A. flavus* was very sensitive to culture pH, optimal pH control strategy throughout the fermentation is not the main requirement to obtain high kojic acid production. In general, kojic acid fermentation with two-phase pH control strategy as proposed in this study produced comparable yield and productivity to that obtained in fermentation without pH control. Although *A. flavus* was capable to utilise gelatinised sago starch for production of kojic acid, the production was reduced with increasing scale of the fermenter due to the high viscosity of the medium that caused improper mixing and reduced mass transfer rate. High DOT (>80%) during growth phase is required to produce cell mycelia with high ability in synthesising kojic acid. However, this was not possible for batch fermentation in stirred tank fermenter using gelatinised sago starch. Fed-batch fermentation has been proposed and successfully used for improvement of kojic acid production using sago starch as substrate. Since starch is a low carbon source, and kojic acid production was high, *A. flavus* strain 44-1 holds promise for the development as an industrial strain. This is the first report appeared in the literature on high kojic acid production using sago starch as substrate.

The cell-bound enzymes relevant to kojic acid synthesis were capable to transform various sugars (glucose, sucrose and fructose) to kojic acid in a resuspended cell system. This method of production was capable to produce significantly high kojic acid free from pigments. This biotransformation process has an advantage in reducing the cost of product recovery and repeated use of the cell mycelia to increase the overall productivity. The important parameters for scaling-up of batch kojic acid fermentation have also been identified. Preliminary production cost evaluation to identify the economic feasibility of kojic acid production by the developed process has also been carried out. To our knowledge, models that can be used to described kojic acid fermentation are not available in the literature. In this study, an unstructured model based on logistic and Luedeking-Piret equations was proposed to describe growth, substrate consumption and kojic acid production by *Aspergillus flavus* Link strain 44-1 in batch fermentation using various carbon sources. The models showed that kojic acid production was non-growth associated. The proposed models may be used for simulation and optimisation of the process.

Very acidic initial pH conditions (2-3) is important for high yield of kojic acid production using various carbon sources even though excellent growth was observed at higher pH. Low culture pH was the optimal conditions for the enhancement of enzymes related to kojic acid synthesis as well as the efficiency of the mycelia for the conversion of glucose to kojic acid during the production phase. It is interesting to note that the variation of pH greatly influenced the ability of cell in producing kojic acid mainly during growth phase. Kojic acid production (23.5 g/L) using 100 g/L gelatinised sago starch in a shake flask was comparable to fermentation of glucose (31.5 g/L) and starch hydrolysate (27.9 g/L) but in the 50 L fermenter was greatly reduced due to non-optimal aeration conditions. For the improvement of kojic acid production using gelatinised sago starch in the fermenter, fed-batch fermentation was applied. An addition of a large volume of concentrated sago starch (140 g/L) to 48 h culture with initial starch concentration of 60 g/L sago starch produced maximum kojic acid (16.43 g/L) of about 4 times higher as compared to batch fermentation of 100 g/L sago starch. Further improvement of kojic acid production was obtained by adding small volume of concentrated starch (140 g/L) intermittently at 48 h intervals to the culture with initial starch concentration of 60 g/L. By using this technique, the dissolved oxygen can be controlled at high levels (40 – 50% saturation) during active growth phase, which is the required condition for the enhancement of α-amylase secretion and also for the production of cell mycelia with higher ability in synthesising kojic acid. The yield (0.36 g kojic acid/g starch) and productivity (0.083 g/L.h) obtained in the fed-batch fermentation using gelatinised sago starch were comparable to the batch fermentation using glucose.

Conclusions

Kojic acid fermentation by *A. flavus* can be divided into two phases; growth and production phase. From the modelling, it was found that kojic acid production by *A. flavus* was non-growth associated process. The kinetic parameter values for each fermenter were calculated from the modelling and they can be used to verify the experimental data using various types and concentration of carbon source. Kojic acid production (23.5 g/L) using 100 g/L sago starch in a shake flask was comparable to fermentation of glucose (32.5 g/L) and starch hydrolysate (27.9 g/L) but in the 8 L and 50 L fermenter kojic acid production was greatly reduced due to non-optimal aeration conditions. Fed-batch fermentation with intermittent feeding of concentrated sago starch (140 g/L) can be employed to improve direct fermentation of sago starch to kojic acid by about 4 times higher as compared to batch fermentation. *A. flavus* was also capable to utilise sucrose for kojic acid fermentation where the highest production (40.23 g/L) in 2 L fermenter was obtained at 150 g/L sucrose. Kojic acid production (10.25 g/L) was greatly reduced in fermentation using fructose as the sole carbon source. Scaling-up based on a constant impeller tip speed (1.65 m/s) together with optimal DOT and pH control strategies was successfully used for kojic acid fermentation in 50 L fermenter using glucose and sucrose as carbon sources. The culture pH during growth phase influenced the performance of kojic acid fermentation to a further extend than did the pH during the production phase. The fermentation without pH controlled (started with an initial culture pH 3) showed higher kojic acid production than single-phase pH controlled fermentation at a range of pH 2.2 – 4.0. Comparable kojic acid production to fermentation without pH controlled was obtained in two-phase pH controlled fermentation (started with initial culture pH, without control during growth phase and switched to 3 during production phase). Efficient conversion of glucose to kojic acid was achieved in a resuspended cell system, in a solution containing only glucose with citrate buffer at pH 3.5 and 30°C. The rate of glucose conversion to kojic acid was increased with increasing glucose concentration up to 100 g/L, suggesting that the biotransformation of glucose to kojic acid by the cell-bound enzymes followed the Michaelis-Menten enzyme kinetic models. The value of K_m and V_{max} for the reaction, as determined by using Langmuir plot, was 10.042 g/L glucose and 0.076 g/L.h, respectively.

Benefits from the study

1. Efficient and high productivity kojic acid fermentation using local sources has been developed.

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Expertise Development

	Name of Graduate	Degree Awarded	Field of Expertise	Graduation Year
1	Rosfarizan Mohamad	Phd	Fermentation Technology	2001
2	Madihah Md Salleh	Msc	Biotechnology	1996
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IRPA Project number: 03-02-04-0109
UPM Research Cluster : BAB
Project Leader assoc. Prof. Dr arbakariya bin ariff