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Kinetics of kojic acid fermentation by *Aspergillus flavus* using different types and concentrations of carbon and nitrogen sources

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Kinetics of kojic acid fermentation by *Aspergillus flavus* Link 44-1 using various sources of carbon [glucose, xylose, sucrose, starch, maltose, lactose or fructose] and nitrogen [NH_4Cl, $(NH_4)_2S_2O_8$, $(NH_4)_2NO_3$, yeast extract or peptone] were analyzed using models based on logistic and Luedeking–Piret equations. The highest kojic acid production (39.90 g l^{-1}) in submerged batch fermentation was obtained when 100 g l^{-1} glucose was used as a carbon source. Organic nitrogen sources such as peptone and yeast extract were favorable for kojic acid production as compared to inorganic nitrogen sources. Yeast extract at 5 g l^{-1} was optimal. The optimal carbon to nitrogen (C/N) ratio for kojic acid fermentation was 93.3. In a resuspended cell system, the rate of glucose conversion to kojic acid by cell-bound enzymes increased with increasing glucose concentration up to 70 g l^{-1}, suggesting that the reaction followed the Michaelis–Menten enzyme kinetic model. The value of K_m and V_{max} for the reaction was 18.47 g l^{-1} glucose and 0.154 g l^{-1} h^{-1}, respectively. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 20–24.

Keywords: kojic acid; *Aspergillus flavus*; fermentation kinetics; resuspended cell system; C/N ratio

Introduction

Attention to kojic acid (5-hydroxy-2-hydroxymethyl-γ-pyrone) production has increased due to its numerous applications in several fields [6,7,14]. Kojic acid is produced industrially by aerobic fermentations using *Aspergillus* spp. Among them, *A. flavus, A. oryzae* and *A. parasiticus* produce large amounts of kojic acid [2,10,12]. Studies on the mechanism of kojic acid production from various carbon sources have revealed that it is a secondary metabolite synthesized via phosphorylated intermediates [4]. Although work on optimization of medium composition has been carried out [10,11,18], details about the nutrient requirements which stimulate kojic acid production have not yet been quantified. The precise levels of carbon and nitrogen that lead to inhibition or enhancement of kojic acid synthesis are not yet identified. It is well known that variations of the carbon to nitrogen (C/N) ratio in the medium greatly influence the metabolite production of many fermentation processes. However, the effect of C/N ratio on the performance of kojic acid fermentation is not available in the literature.

For the development of a commercially feasible fermentation process for large-scale production, improvement of yield and overall productivity are essential. Various methods of optimization, such as experimental design, mathematical methods and kinetics models can be used to improve fermentation processes. Our research focussed on the optimization of kojic acid fermentation using experimental design and modelling. The present study investigated the effect of different types of carbon and nitrogen sources on kojic acid production by *A. flavus* Link 44-1 in shake flask culture. Experimental data obtained were analyzed using kojic acid fermentation models for the calculation of kinetic parameters. The kinetics information may allow better understanding of the process and may also be used for optimization. The relationship between C/N ratio and the performance of the fermentation is also presented and discussed.

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Materials and methods

The fungus *A. flavus* Link 44-1 was used. The methods pertaining to the preparation of slants for spore production and inoculum preparation have been reported [1]. A standard inoculum of 3.5×10^4 spores per milliliter medium was used in all experiments.

The experiment to investigate the feasibility of using different carbon sources (glucose, xylose, sucrose, starch, maltose, lactose and fructose) for kojic acid fermentation was carried out using 100 g l^{-1} of each carbon source and 5 g l^{-1} yeast extract as the sole nitrogen source. To investigate the effect of different nitrogen sources, 100 g l^{-1} glucose was used as a carbon source and the concentration of each nitrogen source was 5 g l^{-1}. The effect of glucose concentration on growth of the fungus and kojic acid production was studied using 5 g l^{-1} yeast extract as the sole nitrogen source. One hundred grams per liter glucose was used as carbon source in the experiment to investigate the effect of yeast extract concentration. The same salt composition (1 g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSO_4{\cdot}7H_2O$) was used in all media, pH of which was adjusted to 3.0 by adding either 1.0 N HCl or 1.0 N NaOH.

All submerged batch fermentations were carried out using 250-ml Erlenmeyer flasks containing 100 ml medium. After inoculation with spores, the flasks were incubated at 30°C on a rotary shaker agitated at 250 rev/min. Kojic acid production by *A. flavus* was also studied using a resuspended cell system; the procedure, cultural conditions and kojic acid determination have been described previously [1,2]. Glucose was determined enzymatically by glucose oxidase using SIGMA Diagnostics Glucose (Trinder) reagent (Catalog number 315-100); the absorbance was measured at 505 nm. The kinetic models for cell growth, substrate consumption and product formation based on logistic and Luedeking–Piret equations as described by Ariff *et al.* [1] were used. The kinetic models are:

Submerged batch fermentation

$$\text{Cell growth}: dX/dt = [\mu_{\max}(1 - X/X_{\max})]X \quad (1)$$

$$\text{Substrate consumption}: -dS/dt = \alpha(dX/dt) + \beta X \quad (2)$$

$$\text{Product formation}: dP/dt = m(dX/dt) + nX \quad (3)$$

Resuspended cell system

$$\text{Substrate consumption}: -dS/dt = \beta X \quad (4)$$

$$\text{Product formation}: dP/dt = nX \quad (5)$$

Results and discussion

Effect of carbon source

Very high maximum cell concentrations (12–15 g l^{-1}) were obtained during kojic acid fermentation when glucose, xylose, sucrose, starch or maltose were used as carbon source (Table 1). With lactose or fructose, the maximum concentrations of cells were 4.4 g l^{-1} and 6.34 g l^{-1}, respectively. The highest kojic acid production was found with monosaccharides (glucose and xylose), with maximum kojic acid concentrations of 39.9 and 35.1 g l^{-1}, respectively. The fungus also used starch, sucrose and maltose as carbon sources. They produced maximum kojic acid concentrations of 23.14, 22.98 and 11.13 g l^{-1}, respectively. Even though high cell concentrations were achieved in fermentations using starch and sucrose (15.02 and 14.83 g l^{-1}, respectively), the efficiencies of kojic acid production ($Y_{P/X}$) were lower than for glucose and xylose (3.44, 2.79 g cell^{-1}, respectively). Glucose was the best carbon source for kojic acid production with the highest yield (0.998 g kojic acid g carbon^{-1}) and productivity (0.083 g l^{-1} h^{-1}). Similar results were reported by Kitada *et al.* [11] who found the best carbon sources for kojic acid production by *A. oryzae* to be glucose and xylose. It has also been reported that kojic acid is formed directly from glucose involving a multistep enzyme reaction without any cleavage into small fragments [4]. Kojic acid production was very low when fructose or lactose were used as carbon sources. Fructose did not support as high kojic acid production as other monosaccharides; this may be due to the possibility that fructose in furanose form was not suitable for direct conversion to kojic acid [15]. This result can also be correlated to the requirement of C6 precursor in the pyranose form for direct conversion of kojic acid [4,5,11].

Effect of nitrogen source

The types of nitrogen source used greatly influenced both growth and kojic acid production (Table 1). High maximum cell concentration ($X_{\max}$) and kojic acid production were obtained when organic nitrogen sources (yeast extract and peptone) were used. Growth of *A. flavus* was very poor when inorganic nitrogen sources were used. However, among the inorganic nitrogen sources tested, only $(NH_4)_2NO_3$ supported kojic acid production, yielding a final concentration of 12.0 g l^{-1} after 504 h of fermentation. Yeast extract gave the highest maximum kojic acid concentration (39.9 g l^{-1}) and overall productivity (0.083 g l^{-1} h^{-1}). In terms of yield, fermentation using peptone gave a slightly higher $Y_{P/N}$ value as compared to fermentations using yeast extract as the nitrogen source.

It has been reported previously that cell development and kojic acid production were higher in fermentations using organic nitrogen sources compared to fermentations using inorganic nitrogen sources [11,12]. Several enzymes such as glucose-6-phosphate dehydrogenase, hexokinase and gluconate dehydrogenase are involved in the biosynthesis of kojic acid [5]. Free amino acids may be required for the enhancement of enzymes relevant to kojic acid synthesis. Yeast extract was the most favorable nitrogen source [5,19]. The use of NH^{4+} ions as on inorganic nitrogen source may repress enzymes associated with kojic acid synthesis.

Table 1 Effect of different carbon and nitrogen sources on growth of *A. flavus* and kojic acid production

Carbon or nitrogen source	$X_{\max}$ (g l^{-1})	$P_{\max}$ (g l^{-1})	$Y_{P/X}$ (g kojic acid g cell^{-1})	$Y_{P/S}$ (g kojic acid g carbon^{-1})	$Y_{P/N}$ (g kojic acid g nitrogen^{-1})	Productivity (g l^{-1} h^{-1})
Carbon source (100 g l^{-1})						
Glucose	11.59	39.90	3.44	0.998	–	0.083
Xylose	12.60	35.10	2.79	0.878	–	0.070
Sucrose	14.83	22.98	1.55	0.544	–	0.046
Fructose	6.34	4.10	0.64	0.102	–	0.008
Lactose	4.40	0.0	0.0015	0.000	–	0.000
Maltose	12.20	11.13	0.91	0.264	–	0.022
Starch	15.02	23.14	1.54	0.525	–	0.046
Nitrogen source (5 g l^{-1})						
Yeast	11.59	39.90	3.44	–	79.80	0.083
Peptone	14.00	36.40	2.60	–	90.85	0.072
NH_4Cl	2.90	0.012	0.004	–	0.008	0.000024
$(NH_4)_2S_2O_8$	4.00	0.06	0.02	–	0.98	0.00012
$(NH_4)_2NO_3$	8.00	12.00	1.51	–	13.79	0.024
$(NH_4)_2SO_4$	4.20	0.05	0.013	–	0.00	0.000099

Table 2 Comparison of the performance and the kinetic parameter values of kojic acid production in batch fermentation at different glucose concentrations

Kinetic parameter values	Glucose concentration (g l^{-1})				
	50	80	100	150	200
μ_{max} (h^{-1})	0.073	0.076	0.075	0.069	0.068
X_{max} (g l^{-1})	10.34	12.14	11.59	15.02	16.18
X_0 (g l^{-1})	0.10	0.1	0.10	0.10	0.10
P_{max} (g l^{-1})	16.47	38.54	39.90	35.94	35.89
P_0 (g l^{-1})	0.10	0.10	0.10	0.10	0.10
α (g glucose g $cell^{-1}$)	1.91	2.30	2.55	4.52	6.45
β (g glucose g $cell^{-1}$ h^{-1})	0.0098	0.0092	0.0126	0.0095	0.0059
m (g kojic acid g $cell^{-1}$)	0	0	0	0	0
n (g kojic acid g $cell^{-1}$ h^{-1})	0.0040	0.0065	0.0090	0.0060	0.0045
$Y_{P/X}$ (g kojic acid g $cell^{-1}$)	1.59	3.17	3.44	2.39	2.22
$Y_{P/S}$ (g kojic acid g $carbon^{-1}$)	0.82	1.20	1.00	0.60	0.45
$Y_{X/S}$ (g cell $carbon^{-1}$)	0.52	0.38	0.29	0.25	0.20
Overall productivity (g l^{-1} h^{-1})	0.033	0.076	0.080	0.071	0.071

NH^{4+} ions have a repressive effect on the production of glucoamylase [3] and protease [9]. A negative effect of NH^{4+} has also been observed on the production of antibiotics [13] and the polysaccharide, pullulan [16], compounds not directly related to nitrogen metabolism. As NH^{4+} ion is readily absorbed, this effect could be due to depletion of precursor pools or cofactor supplying the relevant pathways, in conditions where cell growth is favoured [17]. A higher NH^{4+} availability could favor accumulation of an essential compound that effects repression. Control mechanisms of gene expression involving wide domain regulatory genes and/or integrator genes could be involved in the repressive mechanism as observed in protease production [9].

Effect of glucose concentration

The maximum cell concentration increased with increasing glucose concentration (Table 2). However, the highest kojic acid production (39.9 g l^{-1}) was obtained with 100 g l^{-1} glucose. A slight inhibition in kojic acid production was observed in fermentations using high glucose concentrations (150–200 g l^{-1}). Glucose at 100 g l^{-1} [11,19] and 148 g l^{-1} [18] were optimal for the production of kojic acid by *A. oryzae*.

Kinetic parameters (Table 2) calculated using kojic acid fermentation models (Equations 1–3) were used to verify the experimental data for fermentation using different concentrations of glucose. The maximum specific growth rate of *A. flavus* (μ_{max}) was not significantly different at glucose concentrations between 50 to 100 g l^{-1}. A slight reduction in μ_{max} was observed at higher glucose concentrations (150–200 g l^{-1}). For all fermentations, the value of the growth-associated rate constant (m) for kojic acid was zero, suggesting that the production of kojic acid is a non-growth-associated process. The non-growth-associated rate constant (n) for kojic acid was highest at 100 g l^{-1} glucose. The value of the growth-associated rate constant for glucose consumption (α) increased with increasing glucose concentration, though the value of the non-growth-associated rate constant (β) was highest at 100 g l^{-1} glucose. These observations are consistent with the experimental data for the fermentations using high glucose

Table 3 Comparison of the performance and the kinetic parameter values of batch fermentations using the optimal concentration of glucose (100 g l^{-1}) and different concentrations of yeast extract

Kinetic parameter values	Yeast extract concentration (g l^{-1})				
	2	5	10	15	20
μ_{max} (h^{-1})	0.073	0.075	0.045	0.045	0.038
X_{max} (g l^{-1})	11.6	11.6	26.5	26.9	37.0
X_0 (g l^{-1})	0.10	0.10	0.10	0.10	0.10
P_{max} (g l^{-1})	32.1	39.9	2.4	0.011	0
P_0 (g l^{-1})	0.10	0.10	0.10	0.10	0.10
α (g glucose g $cell^{-1}$)	1.50	2.55	2.39	2.89	2.20
β (g glucose g $cell^{-1}$ h^{-1})	0.0154	0.0126	0.0042	0.0035	0.0034
m (g kojic acid g $cell^{-1}$)	0	0	0	0	0
n (g kojic acid g $cell^{-1}$ h^{-1})	0.0055	0.009	0	0	0
$Y_{P/X}$ (g kojic acid g $cell^{-1}$)	2.77	3.44	0.09	4.1×10^{-4}	0
$Y_{P/N}$ (g kojic acid g $nitrogen^{-1}$)	160.75	77.82	2.38	7.3×10^{-3}	0
$Y_{X/N}$ (g cell g $nitrogen^{-1}$)	58.1	23.48	26.52	17.93	18.52
Overall productivity (g l^{-1} h^{-1})	0.064	0.080	0.0048	2×10^{-5}	0

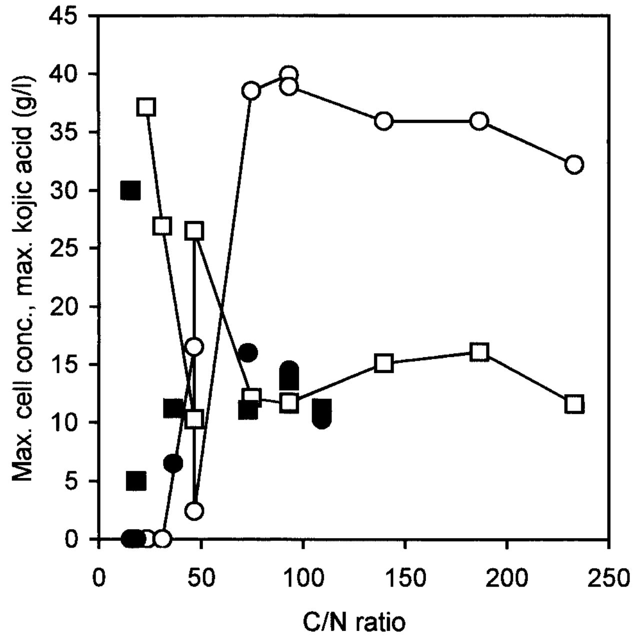

Figure 1 The relationship between C/N ratio and kojic acid production in submerged batch fermentation by *A. flavus*. Symbols represent (○) kojic acid; (□) cell concentration. Open symbols are for the data obtained in this study and closed symbols are for data of Kitada *et al.* [11].

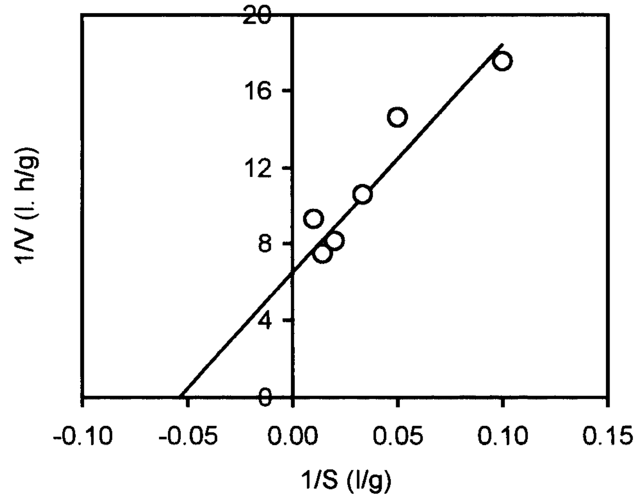

Figure 2 Double reciprocal plot of the Michaelis–Menten equation for the conversion of glucose to kojic acid by biomass of *A. flavus*.

concentrations (150–200 g l^{-1}), which indicated that a large amount of glucose was consumed during the growth phase, but a small amount of glucose was converted to kojic acid during the non-growth phase. A high glucose consumption rate was observed during the non-growth phase of the fermentation using 100 g l^{-1} glucose, with a high kojic acid production. Lower kojic acid production in the fermentations using lower glucose concentrations (50–80 g l^{-1}) may be due to lower glucose concentrations remaining in the culture after growth reached a stationary phase though the mycelia still have a high ability to synthesize kojic acid. Another reason may be the accumulation of other organic acids or other unknown substances which may have inhibited the production of kojic acid. Under glucose-depleted conditions, mycelia of *Aspergillus* spp. degraded kojic acid to other compounds such as oxalic and acetic acids [5,8].

Effect of yeast extract concentration

The performance and kinetic parameter values calculated using kinetic models (Equations 1–3) for fermentations using different concentrations of yeast extract are shown in Table 3. Maximum cell concentration (X_{max}) attained during the fermentation increased with increasing yeast extract concentration. However, kojic acid production was greatly influenced by the concentration of yeast extract in the medium. The highest kojic acid production was obtained in the fermentation using 5 g l^{-1} yeast extract. Kojic acid production was markedly lower with 10 g l^{-1} yeast extract. Kojic acid was not produced at yeast extract concentrations higher than 15 g l^{-1}. Although production in the fermentation using 2 g l^{-1} yeast extract was about 20% lower than that obtained in the fermentation using 5 g l^{-1} yeast extract, the yield based on total nitrogen consumed was two times higher. However, the overall productivity for both fermentations was about the same. Kitada *et al.* [11] reported that 5 g l^{-1} yeast extract was the optimal concentration for kojic acid production (0.80 g l^{-1}) by *A. oryzae*, though production was much lower than that obtained in this study. For fermentations using peptone, the optimal production was obtained at 4.8 g l^{-1} [18].

Table 4 Comparison of the performance and the kinetic parameter values of kojic acid production in a resuspended cell system using different concentrations of glucose

Kinetic parameter values and performance	Glucose concentration (g l^{-1})							
	10	30	50	70	100	150	200	250
Rate of reaction, V (g kojic acid l^{-1} h^{-1})	0.057	0.094	0.123	0.134	0.097	0.079	0.083	0.075
n ($\times 10^{-2}$ g kojic acid g cell^{-1} h^{-1})	1.02	1.68	2.19	2.39	1.73	1.41	1.48	1.35
β (g glucose g cell^{-1} h^{-1})	0.018	0.028	0.032	0.040	0.023	0.027	0.059	0.075
P_{max} (g l^{-1})	8.23	27.53	34.23	42.1	42.02	42.75	42.63	26.98
Amount of glucose consumed during the reaction (g l^{-1})	10	30	50	70	75.46	86.28	67.98	89.38
Process time, t (h)	144	288	288	336	576	528	528	528
$Y_{P/S}$ (g kojic acid g glucose^{-1})	0.823	0.918	0.685	0.601	0.420	0.285	0.213	0.108
Overall productivity (g l^{-1} h^{-1})	0.057	0.096	0.123	0.125	0.073	0.081	0.081	0.051

In fermentations with high yeast extract concentrations, more than 90% of the glucose was consumed for cell development during growth, with less than 10% remaining for conversion to kojic acid during the production phase. This is a possible reason for lower kojic acid production in fermentations using high concentrations of yeast extract. Growth was enhanced and kojic acid production was greatly reduced in kojic acid fermentations by *A. oryzae* using yeast extract concentrations higher than 5 g l^{-1} [10,11]. A high amount of yeast extract added to the culture during fed-batch fermentation was used mainly for cell growth and inhibited kojic acid production [2]. The maximum specific growth rate (μ_{max}) was reduced with increasing yeast extract concentrations. For all fermentations, the value of m is zero. The value of n is higher for the fermentation using 2 g l^{-1} yeast extract compared to the fermentation using 5 g l^{-1} yeast extract. The value of n for fermentations using 15 and 20 g l^{-1} yeast extract is zero, indicating that kojic acid was not produced during the fermentation. However, the value of β is not zero for fermentations using 15–20 g l^{-1} yeast extract, suggesting that glucose consumed during non-growth phase may be used for production of other organic acids or other compounds and not for kojic acid.

Effect of C/N ratio

Figure 1 shows the effect of C/N ratio; the figure also includes data of Kitada *et al.* [11]. It is apparent that there is a critical C/N ratio for growth and kojic acid production. Growth was limited at a C/N ratio above 75. Highest kojic acid production was obtained at a C/N ratio of 93.3 and was much lower at a C/N ratio higher than 74.6. This is in agreement with the results of Kitada *et al.* [11] who showed that optimal kojic acid production was obtained at a C/N ratio between 75 and 100. However, a slightly higher C/N ratio (113.3) for optimal kojic acid production rate was reported by Takamizawa *et al.* [18]. In both cases, peptone was used as nitrogen source. This suggests that kojic acid production was enhanced in nitrogen-limited fermentations and a large amount of the carbon source remained after growth reached a maximum for conversion to kojic acid by the cell-bound enzymes. In order to improve kojic acid production, the amount of nitrogen source added to the medium must be increased with the amount of carbon source at an optimal C/N ratio.

Kojic acid production in a resuspended cell system

Kojic acid production increased while glucose concentration decreased linearly with incubation time, suggesting that the cell bound enzyme system for kojic acid synthesis was stable for prolonged incubation. The kinetic values of the production in a resuspended cell system as calculated according to Equations 4 and 5 are shown in Table 4. The rate of kojic acid production (V) increased with increasing glucose concentration up to 70 g l^{-1}. A reduced production rate was observed at glucose concentrations above 100 g l^{-1}, though the final kojic acid concentrations for glucose concentrations between 70–200 g l^{-1} were not significantly different. The yield of kojic acid based on glucose consumed ($Y_{P/S}$) decreased gradually with increasing glucose concentration. However, the highest productivity was obtained at 70 g l^{-1} glucose. A drastic reduction in kojic acid production at 250 g l^{-1} glucose was observed, indicating that the enzyme reaction was inhibited at high glucose concentrations.

Since the rate of the reaction increased with increasing substrate (glucose) concentration, it is suggested that kojic acid production in the resuspended cell system followed the Michaelis–Menten enzyme kinetic model. The double reciprocal plot (Figure 2) of data from the experiment using 10–70 g l^{-1} glucose, indicated that the biotransformation of glucose to kojic acid by the cell-bound enzyme of *A. flavus* has V_{max} and K_m values of 0.154 g l^{-1} h^{-1} and 18.47 g l^{-1} glucose, respectively.

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