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Standard Review

Kojic acid: Applications and development of fermentation process for production

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Kojic acid, 5-hydroxy-2-hydroxymethyl-γ-pyrone, has many potential industrial applications. In this review, the properties and diverse applications of kojic acid in industries are described. The review also discusses the advance in kojic acid fermentation, focusing on the process development in micro-organisms and strain selection, medium and culture optimization, as well as fermentation modes for commercially viable industrial scale production. The performances of various fermentation techniques that have been applied for the production of kojic acid are compared, while the advantages and disadvantages of each technique are discussed in this paper.

Key words: Kojic acid, mild antibiotic, anti-browning agent, tyrosinase inhibitor, submerged fermentation, resuspended cell system, surface culture.

INTRODUCTION

Kojic acid, which is an organic acid, is produced biologically by different types of fungi during aerobic fermentation using various substrates (Kitada et al., 1967; Ariff et al., 1996; Wakisaka et al., 1998; El-Aasar, 2006). The name 'kojic acid' (which was originally known as Koji acid) was derived from "Koji", the fungus starter or inoculum used in oriental food fermentations for many centuries. Its chemical structure was then extensively investigated and defined as 5-hydroxy-2-hydroxymethyl-γ-pyrone (Yabuta, 1924). Chemical synonyms of kojic acid are known as 5-hydroxy-2- hydroxymethyl-4H-pyran-4-one (Nandan and Polasa, 1985) and 5-hydroxy-2-hydroxymethyl-4-pyrone (Kahn et al., 1995).

The market for kojic acid has been developed for some 40 years since 1955, where Charles Pfizer and Company, USA, announced the first attempt to manufacture this organic acid. The company patented the methods for the production of kojic acid and its recovery, as well as the preparation of derivatives usable as pesticides. However, there was no urgent commercial use for this compound at that time until a rapid growth occurred in various industries recently. In short, the interest in kojic acid is increasing enormously with a growing presence in industries related to its applications, especially in cosmetic industry (Brtko et al., 2004; Bentley, 2006). Although, kojic acid has been industrially produced and applied for some time, it is still extensively studied. Two main areas are normally considered; those associated with the strain development, and those concerned with the development of fermentation process. Kojic acid is produced by *Aspergillus* spp. and *Penicillium* spp., belonging mainly to the *flavus-oryzae-tamarii* groups. Among them, *A. flavus* (Basappa et al., 1970; Ariff et al., 1996), *A. oryzae* (Kwak and Rhee, 1992; Takamizawa et al., 1996), *A. tamarii* (Gould, 1938) and *A. parasiticus* (Nandan and Polasa, 1985; Coupland and Niehaus, 1987; El-Aasar, 2006) were reported to have the ability to produce large amounts of kojic acid. Although, several potential kojic acid producing strains have been isolated, very little attention has been paid to the improvement of the strains, either through mutation or genetic engineering techniques. Details of industrial techniques of kojic acid fermentation are rarely revealed since they comprise the proprietary know-how of each producing company. This review describes and discusses the properties and potential applications of kojic acid, as well as the development of kojic acid fermentation through both approaches, namely microbiology and pro-

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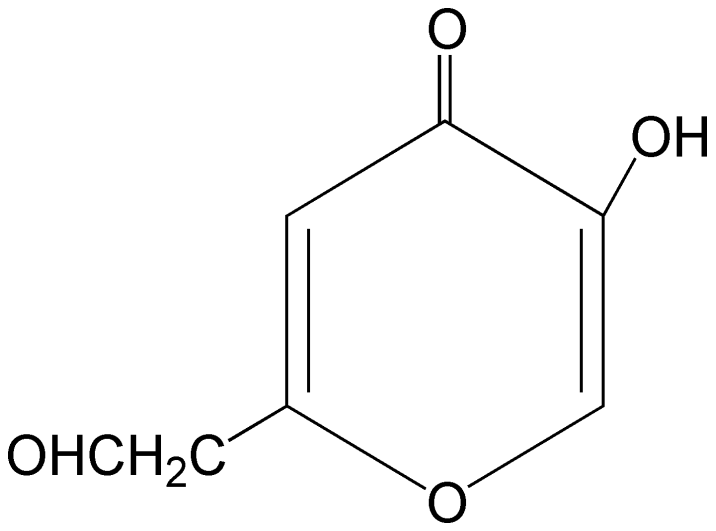

Figure 1. The chemical structure of kojic acid.

cess engineering.

The properties of kojic acid

Kojic acid crystallises in the form of colourless, prismatic needles that sublime in vacuum without any changes. Meanwhile, the melting point of kojic acid ranges from 151°C - 154°C (Ohyama and Mishima, 1990). Kojic acid is soluble in water, ethanol and ethyl acetate. On the contrary, it is less soluble in ether, alcohol ether mixture, chloroform and pyridine (Wilson, 1971). The molecular weight of kojic acid, as determined by the cryoscopic method for a formula of $C_6H_6O_4$, is 142.1 (Uchino et al., 1988). Kojic acid has a maximum peak of ultraviolet absorption spectra at 280 -284 nm (Choi et al., 2002; Watanabe-Akanuma et al., 2007).

The chemical structure of kojic acid is shown in Figure 1. Kojic acid is classified as a multifunctional, reactive γ-pyrone with weakly acidic properties. It is reactive at every position on the ring and a number of products which have values in industrial chemistry, such as metal chelates, pyridones, pyridines, ethers, azodyes, mannich base, and the products of cyanoethylation can be formed from kojic acid (Ichimoto et al., 1965; Wilson, 1971). Numerous chemical reactions of kojic acid have been studied over the decades since its isolation. At carbon 5 positions, the hydroxyl group acts as a weak acid, which is capable to form salts with few metals such as sodium, zinc, copper, calcium, nickel and cadmium (Crueger and Crueger, 1984).

THE APPLICATIONS OF KOJIC ACID

The most striking benefit of kojic acid is found in cosmetic and health care industries. It primarily functions as the basic material for the production of skin whitening creams, skin protective lotions, whitening soaps and tooth care products. Kojic acid has the ability to act as the ultra violet protector, whereby, it suppresses hyper-pigmentation in human skins by restraining the formation of melanin through the inhibition of tyrosinase formation, the enzyme that is responsible for skin pigmentation (Ohyama and Mishima, 1990; Noh et al., 2009). Kojic acid has a melanogenesis-inhibitory effect on *in-vitro* living pigment cells. Kojic acid induces a distinct reduction of eumelanin content and its essential precursor monomer, 5,6DHI 2C in hyper-pigmented B16 cells. The melanogenesis inhibitory effect of kojic acid on the ultraviolet-induced hyper-pigmentation and pigmentary disorders of human skins has been reported in some studies (Ohyama and Mishima, 1990; Lee et al., 2006). At present, kojic acid is primarily used as the basic ingredient for excellent skin lightener in cosmetic creams, where it is used to block the formation of pigment by the deep cells on the skins (Masse et al., 2001). Since the incident of skin cancer is increasing rapidly due to exposure towards high ultraviolet radiation of sunlight, currently, this acid is also widely used in cosmetic industry as a skin protective lotion. It is normally used in combination with alpha-hydroxy acid in the formulation of skin whiteners to control lightened freckles and age spots. Hydroquinone has been banned for cosmetic usage in Asia, and it is noted as a possible carcinogenic compound by the Food and Drug Authority (FDA) of USA. This has led to a significant increase in the use of kojic acid as a replacement for hydroquinone that bleaches and possibly damages skins in cosmetic products. In addition, kojic acid and its manganese and zinc complexes can potentially be used as radio protective agents, particularly against γ-ray (Emami et al., 2007).

Recently, methods for the synthesis of various kojic

Table 1. Applications of kojic acid.

Fields	Functions	References
Medical	Antibacterial	Kotani et al. (1976); Nohynek et al. (2004).
	Antifungal	Kayahara et al. (1990); Balaz et al. (1993).
	Pain killer	Beelik (1956).
Food	Flavour enhancers	Wood (1998); Burdock et al. (2001)
	Antioxidant	Chen et al. (1991); Niwa and Miyachi (1986).
	Maltol and ethyl maltol	Tatsumi et al. (1969).
Agriculture	Anti melanosis	Chen et al. (1991); Saruno et al. (1978).
	Insecticide activator	Buchta (1982); Dowd (1988); Dobias et al. (1977).
Cosmetic	Whitening agent	Niwa and Akamatsu (1991); Masse et al. (2001); Bently (2006); Ohyama and Mishima (1990).
	Ultra violet filter	Noh et al. (2009).
	Tyrosinase inhibitor	Niwa and Akamatsu (1991).
	Radical scavenging activity	Emami et al. (2007).
	Radioprotective agent	
Chemistry	Reagent for iron determination	Bentley (1957).
	Synthesis of 2-methyl-4-pyrone	Hasizume et al. (1968).
	Iron chelator	Mitani et al. (2001).
	Kojic acid-chitosan conjugates	Guibal (2004), Synytsya et al. (2008).

acid derivatives, such as kojic acid ester, kojic acid laureate and kojic acid dipalmitate have been reported in many studies (e.g. Brtko et al., 2004; Lee et al., 2006; Khamaruddin et al., 2008; Ashari et al., 2009). These derivatives have been found to improve both the stability and solubility of kojic acid in oily cosmetic products. In addition, the tyrosinase inhibitory activity of kojic acid derivatives, which are synthesized through an ethylene linkage of phosphonate with aldehyde using intermediates derived from kojic acid, is about 8 times more potent than kojic acid (Lee et al., 2006).

Apart from its main application in cosmetics, kojic acid also has many potential industrial applications (Table 1). It is the first pyrone derivative that is chemically used for analytical iron determinations in ores. Metal chelates of kojic acid have been advocated as the source materials giving the controlled release of metallic ions in curing agents or catalysts (Wilson, 1971).

Kojic acid gives a deep red colour with as little as 0.1 ppm ferric ion, and the ferric complex cannot be reversibly oxidised or reduced (Buchta, 1982).

The natural origin of kojic acid, which ensures its non-problematic biodegradation, makes it an attractive basic skeleton for the development of biologically active compound via derivatization. Kojic acid and its derivatives possess antibiotic properties against gram-negative as well as gram-positive micro-organisms (Kotani et al., 1976). The bacterial growth is generally inhibited in the presence of more than 0.5% (w/v) of kojic acid (Beelik, 1956). It is also active against human tubercle bacilli in *in vitro* technique under a variety of conditions, whereby 45 mg/100 mL of kojic acid completely inhibits the surface growth of bacilli (Lee et al., 1950). In addition, kojic acid in the form of azidometalkojates also shows antibacterial and antifungal effects on several species of *Bacillus*, *Staphylacoccus*, *Saccharomyces*, *Aspergillus*, *Rhizopus* and *Fusarium*. Azidometalkojates, in the form of zinc derivatives, also exhibits a certain cytotoxic activity on HeLa tumour cells (Hudecova et al., 1996). Recently, Nohynek et al. (2004) reported that the antibacterial feature of kojic acid, over a few common bacterial strains, is found to be significant only in dilutions of 1:1000 - 1:2000.

Kojic acid and its derivatives (mostly at the 7-iodo kojic acid) have a potent activity against bacteria such as *Staphylococcus aureus* 209 (Kotani et al., 1976). The compound of kojic acid derivatives was also tested for antifungal activities against *Phythium graminicola*, *Fusarium oxysporum* and *Rhizoctonia solani*, which cause seedling blight, fusarium wilt and sheath blight, respectively (Kayahara et al. 1990; Balaz et al., 1993). Besides its antibiotic functions, kojic acid also shows a certain insecticidal activity against *Heliothis zea* and *Spodoptera frugiperda* insects. In addition, it has been

employed as a chelating agent for the production of insecticides (Buchta, 1982; Dowd, 1988). Kojic acid has been shown to inhibit the growth of *Trogoderma* larvae and induce sterility in males and females of this genus (Sehgal, 1976). Furthermore, it also inhibits the development of *Musca domestica* (Beard and Walton, 1969), as well as that of *Drosophila melanogaster* (Dobias et al., 1977). The potential use of kojic acid and its derivatives in humans and/or veterinary medicines as biological active compounds has also been reviewed by Brtko et al. (2004).

Kojic acid is distributed naturally in traditional Japanese food such as miso, soy sauce and sake, thus, endowing these various food types with special tastes, colours and flavours (Wood, 1998). Not only that, kojic acid also acts as a precursor for flavour enhancers (that is**,** maltol and ethyl maltol). It is used in the production of comenic acid, which is an intermediate for the synthesis of maltol (3-hydroxy-2-methyl-γ-pyrone) and its derivatives (Tatsumi et al., 1969). The synthesis of maltol is useful for improving the flavours of various food products and as the ingredient in perfumes and flavours (Ichimoto et al., 1965). The health aspects of kojic acid in food have been evaluated by Burdock et al. (2001). In fact, the researchers suggested that the consumption of kojic acid, at levels normally found in food, does not require a concern for safety.

Uchino et al. (1988) recognised kojic acid for its antispeck activity. It can be used to prevent browning formation (speck) during the storage and processing of raw noodles (uncooked). Besides, it also has an inhibitory effect on polyphenol oxidase in mushrooms (Saruno et al., 1978), apples, potatoes and crustaceans including white shrimps, grass prawns and Florida spiny lobsters (Chen et al., 1991). The inhibitory effect of kojic acid on polyphenol oxidase is associated to the inhibition of melanosis by interfering the uptake of oxygen required for enzymatic browning, and reduction of o-quinones to diphenols to prevent the formation of the final pigment (melanin) or the combination of the above actions.

Kojic acid is recognised as an important intermediate in the production of chemicals that can be used as pharmaceuticals. Novotny et al. (1999) claimed that kojic acid could be used in the preparation of compounds with an anti-neoplastic potential. In addition, the anti-neoplastic activity of kojic acid derivatives is based on various mechanisms of actions on different levels of cellular metabolism and functions, which make this compound useful as a cytotoxic agent. Garcia and Fulton (1996) reported that kojic acid, in combination with glycolic acid and hydroquinone, can be used for the treatment of melasma and related conditions.

The formulation is now available to dermatologists to satisfy the patient's preferences. Although**,** hydroquinone alone is effective and has been available for years, kojic acid has the advantages of being pharmaceutically more stable and also as a tyrosinase inhibitor. Recently, it has been reported that kojic acid can be easily conjugated with chitosan to produce kojic acid-chitosan conjugates, suggesting that kojic acid has a potential use in chemical industry (Guibal 2004; Synytsya et al., 2008). At present, chitosan is well-known for its wide applications in various industries such as food, pharmaceuticals, cosmetics, agriculture and environment (Ravi-Kumar, 2000). Moreover, kojic acid has also been conjugated with amino acids to form conjugates which exhibit a higher tyrosinase inhibition activity and stability than kojic acid alone (Noh et al., 2009).

THE DEVELOPMENT OF KOJIC ACID FERMENTATION

Micro-organisms and strain improvement

In general, *Aspergillu*s spp., which belongs mainly to the *A. tamarii* group, is widely used in fermentation of kojic acid. Kitada et al. (1967)**;** Kwak and Rhee (1991) produced kojic acid using *A. oryzae* and yielded 0.26 g of kojic acid/g glucose. In addition, kojic acid production by *A. parasiticus* and *A. candidus* has also been reported by El-Aasar (2006) and Wei et al. (1991), respectively. In these two cases, the yield was 0.089 g kojic acid/g glucose and 0.3 g kojic acid/g sucrose, respectively. On the other hand, a very high yield (that is, 0.453 g kojic acid/g glucose) was also obtained in the fermentation using *A. flavus* (Ariff et al., 1997; Rosfarizan et al., 2007). Having mentioned this, the risk of aflatoxin production by this strain cannot be ignored. However, Madihah (1996) reported that aflatoxin production by kojic acid producing *A. flavus* can be inhibited by a suitable medium formulation and appropriate culture conditions. In addition, kojic acid and aflatoxin synthesis follow different pathways and therefore, kojic acid is not an intermediate for the synthesis of aflatoxin by *Aspergillus* spp. (Basappa et al., 1970).

Kojic acid producing strains, *A. flavus* has been improved by the monospore isolation method to obtain a stable monokaryotic strain capable of producing a substantially higher amount of kojic acid (19.2 g/L) as compared to the unstable heterokaryons (10.5 g/L) (Rosfarizan et al., 1998). Kojic acid producing strain could also be improved through mutation and genetic recombination techniques (Fantini, 1975; Crueger and Crueger, 1984). Moreover, the improvement of kojic acid production by phototropic *A. oryzae* (green conidia) through mutation has been reported by Demain (1973). The mutation was achieved by producing lysine auxotrophy with yellow conidia, and this was found to produce kojic acid five times higher than the parent strain. A mutant strain of *A. oryzae* MK107-39 was also capable to produce kojic acid by about 7.7 times than its parent strain (Futamura et al., 2001). The mutation of *A. oryzae* ATCC 22788 via NTG treatment and UV irradiation, followed by protoplast was also found to improve kojic acid production (41 g/L) of about 100 times been reported that kojic acid can be easily conjugated

Table 2. Carbon sources for kojic acid production by various types of micro-organism.

Carbon source	Number of carbon atom	Micro-organism	References
Ethanol	2	*A. oryzae*	Basappa et al. (1970)
Glycine			
Sodium acetate		*A. flavus*	Arnstein and Bentley (1956)
1,3-Dihydroxy-2-propanone	3	*A. oryzae*	Arnstein and Bentley (1956)
Glycerol		*A. tamarii*	Gould (1938)
Tartaric acid	4	*A. oryzae*	Tamiya (1932)
Arabinose	5	*A. flavus*	Arnstein and Bentley (1953a),
Xylose			Basappa et al. (1970)
Fructose	6	*A. tamarii*	Gould (1938)
Glucose		*A. luteo-virescens*	Morton (1945)
		A. oryzae	Arnstein and Bentley (1956),
			Kitada et al. (1967), Takamizawa et al. (1996)
		A. flavus	Ariff et al. (1996), Kwak and Rhee (1991), Bajpai et al. (1982), Wan et al. (2005)
		A. candidus	Wei et al. (1991)
		A. parasiticus	El-Aasar (2006).
Shikimic acid	7	*A. oryzae*	Katagiri and Kitahara (1933)
Maltose	12	*A. oryzae*	Kitada et al. (1967)
Sucrose		*A. tamari*	Marston (1949)
		A. flavus	Rosfarizan and Ariff (2007)
Starch	-	*A. flavus*	Rosfarizan et al. (1998)

OPTIMISATION OF MEDIUM COMPOSITION

Carbon sources

A variety of carbon containing substrates may be used as carbon sources for kojic acid fermentation (Table 2). These substrates include starch, sucrose, maltose, glucose, fructose, mannose, galactose, xylose, arabinose, sorbitol, acetate, ethanol, glycerol and arabinose (Arnstein and Bentley, 1956; Wilson, 1971; Burdock et al., 2001; Rosfarizan and Ariff 2007). 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). Excellent growth was obtained in all types of carbon sources investigated. Nevertheless, kojic acid was not detected in the fermentation using starch, fructose and xylose. The highest yield of kojic acid was obtained 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 that can be obtained from fermentation by various kojic acid-producing strains (Kitada et al., 1967). 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).

On the other hand, Wei et al. (1991) reported that sucrose was a better carbon source than glucose for the production of kojic acid by *A. candidus*, in which the acid yielded 0.5 g kojic acid/g sucrose for the fermentation using sucrose that produced about two times higher than the yield obtained from glucose, that is, 0.25 g kojic acid/g glucose. Generally, polysaccharides with a long chain of carbon sources, such as starch, are considered as poor carbon sources for fermentation of kojic acid. *A. flavus* strain (S33-2), which is capable of growing on cooked starch and producing substantially high level of kojic acid, has been isolated (Rosfarizan et al., 1998). The yield of 0.25 g kojic acid/g starch was obtained in the fermentation using corn starch.

The concentration of glucose, as a carbon source, also greatly influences kojic acid production. Kitada et al. (1967) investigated the effect of glucose concentrations ranging from 25 - 150 g/L on the production of kojic acid by *A. oryzae*. Although, the maximum cell yield was not significantly different at above 50 g/L glucose, the highest kojic acid production (24.2 g/L) was obtained using 100 g/L glucose, giving a yield of 0.24 kojic acid/g glucose. At 25 and 50 g/L glucose, all glucose supplied was consumed for biomass built-up and kojic acid production was delayed. About 50% of the supplied glucose was not consumed during the fermentation using 150 g/L glucose. El-Aasar (2006) also reported that the highest production of kojic acid was obtained in the fermentations which employed either 60 g/L glucose, 40 g/L sucrose and 60 g/L beet molasses with the yields of 0.43 g kojic acid/g

Table 3. Nitrogen sources for kojic acid production by various types of micro-organisms.

Micro-organism	Nitrogen source (g/L)	References	
A. oryzae	$(NH_4)_2SO_4$	0.5	Katagiri and Kitahara (1933)
	Yeast extract	10	Arnstein and Bentley (1953c)
		5	Ogawa et al. (1995)
		5	Kitada et al. (1967)
A. flavus	NH_4NO_3	1.1	May et al. (1931)
	Yeast extract	1	Ariff et al. (1996)
		5	Megalla et al. (1987)
		6	Wan et al. (2005)
A. tamarii	$NaNO_3$	2	Gould (1938)
A. albus	Polypeptone	7	Saruno et al. (1978)
A. candidus	Yeast extract	1	Wei et al. (1991)
A. parasiticus	Peptone	2	Coupland and Niehaus (1987)
	Yeast extract	10	El-Aasar (2006)

glucose, or 0.475 g kojic acid/g sucrose, 0.35 kojic acid g/g molasses, respectively.

Nitrogen sources

Table 3 shows the variation in nitrogen sources chosen for kojic acid fermentation from several strains of *Aspergillus* spp. Kitada et al. (1967) reported that organic nitrogen sources are generally better than inorganic nitrogen sources for kojic acid fermentation. Complex organic nitrogen sources such as peptone and yeast extract may contain vitamins, which act as a precursor for kojic acid production. Furthermore, some organic nitrogen sources have a good buffering system, while inorganic nitrogen sources, such as ammonia, excessively reduce the culture pH during NH_4^+ absorption. Low pH may influence the synthesis of kojic acid during fermentation and inhibit its growth. Yeast extract has been reported to be the most favourable organic nitrogen source for kojic acid production as compared to peptone and polypeptone (Wei et al., 1991; Ariff et al., 1996). However, Kitada et al. (1967) and Coupland and Niehaus (1987) claimed that peptone is better than yeast extract for kojic acid production. The presence of important growth factors, such as vitamins and oligoelements in specific nitrogen sources, also plays an important role in enhancing kojic acid production (Gad, 2003). Coupland and Niehaus (1987) pointed out that the addition of 10 mM $(NH_4)_2SO_4$ and glycerine to a medium containing 2 g/L peptone repressed kojic acid synthesis significantly.

On the other hand, high production of kojic acid (83 g/L), using a mixture of nitrogen source (0.5 g/L yeast extract + 0.75 g/L $(NH_4)_2SO_4$), had been reported by Kwak and Rhee (1992). Megalla et al. (1987) also used a mixture of nitrogen sources (2.0 g/L $(NH_4)_2SO_4$ + 1 g/L yeast extract) to produce a fairly high kojic acid yield (25 g/L) by *A. flavus*. A high kojic acid production (28.4 g/L) was obtained by *A. parasiticus* fermentation utilizing 10 g/L yeast extract, whereas**,** the amount of acid production was greatly reduced with $(NH_4)_2SO_4$ and $NaNO_3$ (El-Aasar, 2006). Hence, NH_4^+ ion has the tendency to lower the culture pH excessively (Kitada et al., 1967).

A medium containing 100 g/L glucose as a carbon source and yeast extract concentrations ranging from 0.5 - 2.5 g/L or peptone concentrations ranging from 1 - 5 g/L as the nitrogen source is normally used in kojic acid fermentation by *Aspergillus* spp. Due to the presence of high concentration of yeast extract or peptone in a medium, glucose cannot to be converted to kojic acid but it is utilised for cell growth instead. Limitation of nitrogen supply is required to limit the growth so that more glucose can be converted to kojic acid. Using 100 g/L glucose, the optimum production of kojic acid was obtained at 0.3 g/L total amino nitrogen (Kwak and Rhee, 1992) and 2 g/L peptone (Kitada et al., 1967). Therefore, nitrogen limited fermentation is essential to limit the growth, so that the excess glucose which still remains in the culture can be converted to kojic acid by the activity of non-growing mycelial cells (Ariff et al., 1996).

C/N ratio

It is important to quantify the variations of carbon to nitrogen (C/N) ratio as it leads to the inhibition and enhancement of kojic acid synthesis, as well as to obtain optimal production. The effects of C/N ratio on the production of kojic acid have been reported by various researchers, and these are summarized in Figure 2. It is apparent that there is a critical C/N ratio for kojic acid production. It can be seen that C/N ratio higher than 100

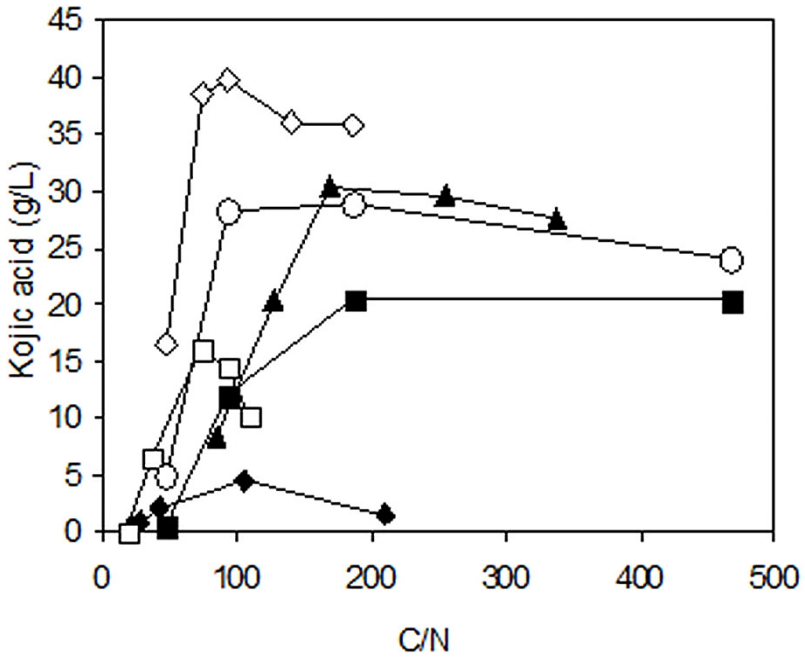

Figure 2. Effects of C/N ratio on the performance of kojic acid fermentation as reported by various researchers.
Note: (▲) Kwak and Rhee (1992); (♦) Coupland and Niehaus (1987); (■) Ogawa et al. (1995); (◊) Madihah et al. (1996); (□) Kitada et al. (1967); (O) Ogawa et al. (1995) MSLC.

is essential for the enhancement of kojic acid production. In most cases, the C/N ratio which is higher than 100 was not found to significantly influence kojic acid production. However, kojic acid production was reduced greatly at C/N ratio lower than 100. From this observation, it can be concluded that nitrogen limited fermentation is essential for kojic acid production. A large amount of carbon sources should be available during the production phase for the conversion to kojic acid by the cell bound enzymes and other activities of non-growing cells.

Minerals

Czapek-Dox medium containing KH_2PO_4, KCl, $NaNO_3$, $MgSO_4$ and $FeSO_4.7H_2O$ is favourable for the growth of kojic acid producing fungus (*A. flavus* and *A. oryzae*) and kojic acid production (Basappa et al., 1970; Wei et al., 1991). However, the medium with carbon and nitrogen sources containing only KH_2PO_4 and $ZnCl_2$ as minerals can also be used for a good growth of *A. parasiticus* and substantially high kojic acid production (Coupland and Niehaus, 1987).

Phosphate is an important nutrient for the growth of most fungi. It is incorporated in molecules such as nucleic acids, phospholipids and sugar phosphate; and plays an essential role in energy metabolism. The concentration of phosphate in the culture broth gives a significant influence on kojic acid production by *A. oryzae* (Arnstein and Bentley, 1953b). Previous studies revealed that high phosphate concentration in the culture broth (that is, 0.55 -13.72 mM) resulted in rapid kojic acid production. Conversely, at lower concentrations of phosphate (that is, 0.0055 - 0.055 mM), the rate of kojic acid was very much lower, while the maximum concentration of kojic acid obtained was about 2 times lower than those obtained in fermentation using high concentrations of phosphate. However, Coupland and Niehaus (1987) reported that the variation in phosphate concentrations ranging from (0.1 - 100 mM) gave no effects on kojic acid production by *A. flavus*.

Inhibitors and stimulants

Some components in a fermentation medium assist to regulate the production of products rather than to support the growth of micro-organisms. Such additives include inhibitors and stimulators, both of which may be used to manipulate the progress of fermentation. Past studies showed that the production of kojic acid by *A. oryzae* was inhibited by metabolic inhibitors such as sodium fluoride, monoiodoacetic acid, sodium arsenate, malonate, potassium cyanide, sodium azide dinitrophenol and pentachlorophenol at concentrations ranging from 10^{-2} to 10^{-4} M (Kitada and Fukimbara, 1971). However, no inhibition

of kojic acid biosynthesis occurred when sodium sulphite was added to the medium. Furthermore, Basappa et al. (1970) reported that malonate and $Na_2S_2O_4$ inhibited the production of kojic acid by *A. flavus*. The inhibition of kojic acid production and glucose consumption by the above mentioned inhibitors was recovered by the addition of intermediates such as succinate, pyruvate and citrate at a concentration of 10^{-2} M to the inhibited culture (Kitada and Fukimbara, 1971). The growth, kojic acid production and glucose consumption by kojic acid producing fungus (*A. flavus, A. oryzae* and *A. parasiticus*) were inhibited by some chlorinated hydrocarbons such as aldrin, DDT and Lindane, which are normally used as pesticides (Nandan and Polasa, 1985).

Megalla et al. (1987) discovered that the addition of copper-monovales-nicotinic acid complex into *A. flavus* culture enhanced kojic acid production by about 47%. In the fermentation using 100 g/L glucose, the highest production of kojic acid was obtained when 75 µg/100 mL copper (I) – B2 complex was added. This compound, which is also known as a chelating agent, is considered to be a heavy metal vitamin derivative, which precipitates in certain enzymatic reactions. Moreover, the researchers also investigated the biochemical effects of the chelating agent on kojic acid biosynthesis and related the results on the model basis of the biosynthesis pathway of kojic acid, as proposed by Bajpai et al. (1981). The emphasis was placed on the enzymes that were dependent on NAD and NADP. The presence of highly reactive NAD and NADP like structures in the medium promoted the action of dehydrogen, which is the main enzyme involved in the biosynthesis of kojic acid.

The synthesis of kojic acid was unaffected by the changes in the concentrations of zinc and ferric ion, although, high concentrations of NaCl (> 100 mM) and NaOAc (1 - 2 mM) inhibited the synthesis (Coupland and Niehaus, 1987). The addition of 1 - 2 g/L cycasin (methyl-azoxymethyl-β-D-glucose), extracted from the *Cycas revoluta* plant, inhibited the spore formation of *A. oryzae* and increased the kojic acid production by about 6-fold (Tadera et al., 1985). Enhanced kojic acid production by *A. flavus* with the addition of methanol in the culture broth had been reported by Madihah et al. (1996). The presence of methanol in the culture might reduce the bubble size in the stirred tank fermenter and increase the oxygen transfer rate, which in turn, enhanced the fermentation performance.

OPTIMISATION OF CULTURE CONDITION

Culture pH

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 Lundjgren, 1985). The effects of initial pH on kojic acid production by *A. parasiticus* were studied by Lin et al. (1976) using yeast extract as the nitrogen source. The researchers reported that the kojic acid production was optimal at two pH values (4.5 and 6.2). Katagiri and Kitahara (1933) found that an initial pH 5 was favourable for the growth of *A. oryzae*, but an initial pH 2.4 was required to enhance the synthesis of kojic acid. This means that**,** the optimal pH for kojic acid synthesis was different with the optimal pH for the growth of kojic acid producing strain. In the replacement culture, the highest conversion of glucose to kojic acid was obtained at a very low pH (1.9) (Wood, 1998). At slightly higher pH than 1.9 (pH 2.2), the conversion of glucose to kojic acid was reduced significantly. Conversely, at very low pH, the metabolism of the fungus might be shunted into another pathway, which led to kojic acid synthesis. Clevstrom and Lundjgren (1985) also pointed out that the formation of kojic acid occurred most readily at low pH values (2 **-** 3). In addition, Wilson (1971) reported that the optimal pH for kojic acid formation was in the range of pH 2 **-** 3, and a small deviation of pH from this range tended to reduce the production sharply. On the other hand, El-Aasar (2006) claimed that the kojic acid production by *A. parasiticus* was optimal at pH 5.

It is important to note that the optimum pH for kojic acid production also depends on the types of carbon or nitrogen used. Kitada et al. (1967) identified that the optimum pH for kojic acid production was in the range of pH 2 **-** 3, when a combination of glucose or sucrose as the carbon source and peptone or yeast extract as the nitrogen source was used. On the other hand, when ammonium nitrate was used as the nitrogen source, the maximum kojic acid production was achieved at pH 3.08. In addition, Basappa et al. (1970) also reported that the pH range for the maximum kojic acid production by *A. flavus* was between 6 **-** 7, when acetate was used as the carbon source.

Aeration and agitation

Fermentations of kojic acid at pilot and industrial scales are usually conducted using stirred tank fermenters to ensure efficient oxygen transfer into the culture. Some studies on the effects of aeration and agitation on kojic acid production in stirred tank fermenters at industrial scale had been conducted by Kitada et al. (1971). The highest kojic acid production (32 g/L) in a 300 L stirred tank fermenter was obtained at 1 vvm and 240 rpm (impeller tip speed = 8.04 m/s) which gave the value of oxygen transfer rate coefficient of 11.2 X 10^{-6} g/mol O_2/min.atm.mL. High kojic acid production was obtained in the fermentation where dissolved oxygen tension (DOT) was controlled at very high level (80% saturation) during an active growth phase, and DOT was then switched to 30% saturation during the production phase (Ariff et al., 1996). High DOT (80% saturation) was requir-

ed during the growth phase for the production enhancement of enzymes responsible for kojic acid synthesis. Glucose was converted to kojic acid by these cell-bound enzymes during the production phase. Very low DOT (30% saturation) should be controlled during the production phase to avoid excessive degradation of kojic acid to other compounds.

FERMENTATION TECHNIQUES

Various fermentation techniques, such as submerged, solid state and surface cultures, and modes of fermenter operation such as batch, fed-batch and continuous, have been developed and used for the improvement of various fermentation processes (Table 4). Nevertheless, very few attempts had been made to produce kojic acid using solid-state fermentation. For example, Kharchenko (1999) reported that a comparatively high yield of kojic acid (8.5 - 9.5 g kojic acid/kg substrate) was obtained in a solid-state fermentation using grains and grain-forages with high amount of proteins and carbohydrates such as maizes, oats, ryes and barley grains. Most reports on kojic acid production available in the literature are related to submerged fermentation. The feasibility of using different types of submerged fermentation techniques and modes of fermenter operation on the improvement of kojic acid fermentation are outlined and discussed below.

Surface culture

Surface culture is known as the technique where molds are grown on a solid or liquid medium without any agitation or shaking of the culture vessel (Wei et al., 1991). Kojic acid production by surface culture of *A. parasiticus* had been reported in several studies (Lin et al., 1976; Nandan and Polasa, 1985). Meanwhile, *A. candidus* ATCC 44054 produced more kojic acid in the surface culture as compared to the cultures shaken at 100 rpm using the same medium (Wei et al., 1991). However, the process parameters such as pH, temperature and dissolved oxygen tension are difficult to control in the surface culture system.

Submerged culture

Submerged culture is widely used for high performance of kojic acid production (Kitada et al., 1967; Wei et al., 1991; Ariff et al., 1996; Ariff et al., 1997). The growth of aerobic micro-organisms in a submerged culture is controlled by the availability of substrates, energy and enzymes. Cultures are always of a heterogeneous nature, whereby the rates of reactions can be limited by the rate of substrate or product transfer at a particular interface. Different techniques of fermentation, such as batch, fed-batch and continuous for the improvement of kojic acid production, are also possible to be applied in submerged fermentation in order to achieve an optimal and economic fermentation process (Kitada et al., 1967; Ariff *et* al., 1996).

A typical time course of batch kojic acid fermentation by *A. flavus* is shown in Figure 3 (Ariff et al., 1996; Ariff et al., 1997). The kojic acid fermentation by *A. flavus* was classified as a non-growth associated process, where the fermentation could be divided into two phases, namely the growth phase and the production phase. The growth normally reached the maximum after 120 h and the production of kojic acid would start after about 48 h **-** 72 h, whereby the production continued almost linearly until the exhaustion of glucose. On the other hand, Kitada et al. (1967) found that kojic acid fermentation by *A. oryzae* was a mixed process, whereby**,** kojic acid was produced during the growth and non-growth phases. After all supplies of glucose had been consumed, kojic acid accumulated in the culture may be utilised by micro-organisms to produce other substances such as oxalic and other acids (Clevstrom and Ljunggren, 1985), resulting in the decrease of kojic acid production. Excessive degradation of kojic acid, at certain conditions, was also observed towards the end of batch fermentation (Kitada et al., 1967; Ogawa et al., 1995; Ariff et al., 1996).

The use of two-stage continuous culture for kojic acid production by *A. oryzae* using peptone as the growth-limiting nutrient had been reported by Kitada and Fukimbara (1970). Slightly lower concentration of kojic acid at steady-state (6 - 7 g/L) in the first and second vessels was obtained as compared to the batch fermentation (9 g/L). The continuous culture is an ideal method of fermentation for the production of microbial biomass and other growth associated processes such as ethanol production rather than for the production of secondary metabolites or non-growth associated processes such as kojic acid.

In kojic acid fermentation, the fungi initially utilize the carbon source for growth and then synthesize the kojic acid in subsequent declining phase and early stationary phase (Kitada et al., 1967). Extending the culture time while the cells are still actively excreting the metabolite or containing a stable mycelial-bound enzyme that is responsible for kojic acid synthesis (Bajpai et al., 1982) could improve kojic acid production. Fed-batch culture had been applied for the production of kojic acid by *A. oryzae* using the membrane surface liquid culture, where powdered glucose was added intermittently to the initial batch fermentation after glucose supply was exhausted (Ogawa et al., 1995). In this technique of fermentation, the production of kojic acid was increased almost linearly up to about 80 g/L after 384 h. In addition, the yield (0.56 g kojic acid/g glucose) and productivity (0.208 g/L.h) obtained from the fed–batch fermentation were higher than that obtained from the batch fermentation (0.45 g kojic acid/g glucose and 0.121 g/L.h, respectively). Fed-

Table 4. Kojic acid production using different fermentation techniques by various kojic acid-producing micro-organisms.

Method	Micro-organism	Carbon source (g/L)	Nitrogen source (g/L)	pH	Yield (g kojic acid/g carbon source)	Productivity (g/L.h)	References
Surface culture	*A. candidus*	Sucrose (200)	Yeast extract (20)	6.5	0.3	0.208	Wei et al. (1991)
	A. tamarii	Glucose (50)	$NaNO_3$ (2)	4.85	0.24	0.081	Gould (1938)
	A. oryzae	Maltose (50)	$(NH4)_2SO_4$ (0.5)	4.5	0.41	0.043	Katagiri and Kitahara (1933)
Submerged culture	*A. flavus*	Glucose (150)	NH_4NO_3 (28)	-	0.57	0.135	May et al. (1931)
		Glucose (100)	Yeast extract (5)	3.5	0.29	0.13	Ariff et al. (1996)
	A. oryzae	Glucose (100)	Yeast extract (6)	5.0	0.41	0.22	Wan et al. (2005)
		Glucose (100)	Peptone (5)	4.0	0.24	0.143	Kitada et al. (1967)
	A. parasiticus	Glucose (100)	Yeast extract (0.5)	6.0	0.51	0.177	Ogawa et al. (1995)
		Glucose (60)	Yeast extract (10)	5.0	0.57		El-Aasar (2006)
Resuspended cell system	*A. flavus*	Glucose (200)	Yeast extract (20)	6.5	0.41	0.107	Bajpai et al. (1982) Rosfarizan and Ariff (2007)
Immobilised cell system	*A. oryzae*	Glucose (100)	Yeast extract (1)	6.0	0.38	0.154	Kwak and Rhee (1991)
Membrane surface liquid culture	*A. oryzae*	Glucose (100)	Yeast extract (1)	6.0	0.31	0.137	Wakisaka et al. (1998)
		Glucose (100)	Yeast extract (0.5)	6.0	0.46	0.121	Ogawa et al. (1995)

batch fermentation was also performed by Ariff et al. (1996) in order to investigate the requirement of yeast extract during kojic acid production by *A. flavus*. Rapid kojic acid production, together with rapid glucose consumption, occurred even though there was a growth and a high nitrogen level was still present in the culture. However, kojic acid production was very much lower than that obtained in nitrogen limited batch fermentation. Reduced kojic acid production in the fed-batch fermentation was due to the high consumption of glucose for the growth during the production phase and less glucose was converted to kojic acid. This result also showed that the growing cells and unlimited nitrogen did not repress the metabolic pathway of kojic acid production. However, active growth limited a turnover of biomass, which in turn, reduced the ability of mycelia to synthesize kojic acid. Limitation of the nitrogen supply was required to limit the growth so that**,** more glucose could be converted to kojic acid. Since mycelial-bound enzyme system involved in kojic acid biosynthetic pathway was stable for a long starvation period (Kwak and Rhee, 1992), efficient utilization of the remaining activities from batch fermentation might be the extension of production with glucose feeding alone. Fed-batch culture by feeding glucose alone should be started after glucose in the culture is almost exhausted. The glucose feeding should be continued until no more glucose could be converted to kojic acid.

The application of fed-batch culture for improvement of kojic acid production by *A. flavus* using sago starch as the carbon source had been reported by Rosfarizan et al. (2002). Improved kojic acid production, in terms of total productivity, could also be achieved using repeated batch fermentation technique (Wan et al., 2005), where the cultivation period could be extended by culture withdrawal (75% of the total volume) and substitution with a fresh medium.

Membrane surface liquid culture

In submerged culture, molds like *A. flavus* grow either in the form of pulpy or pellet-like morphology (Braun and Vecht-Lifshitz, 1994). In the pulpy

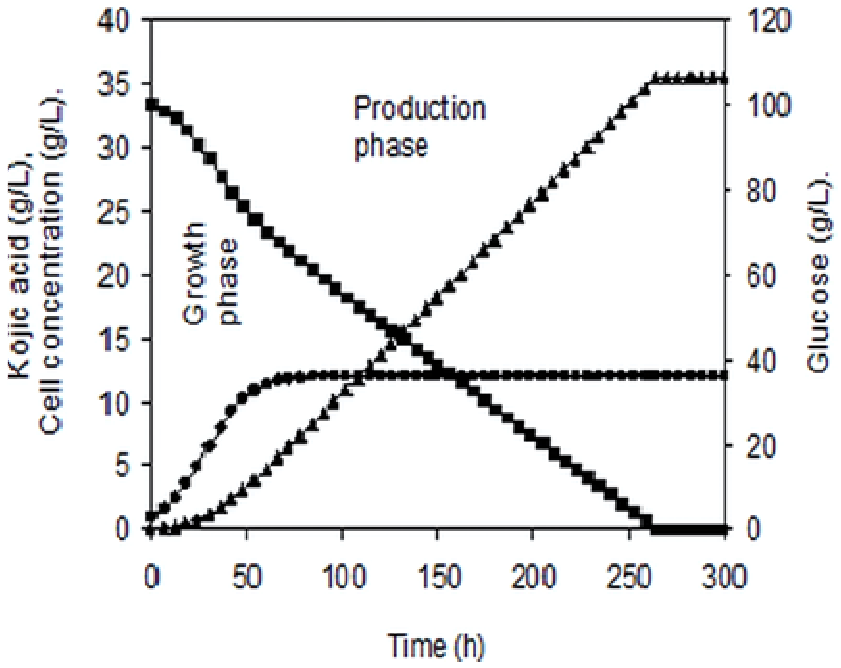

Figure 3. A typical time course of batch kojic acid fermentation by *A. flavus*.
Note: (●) Cell concentration; (■) Glucose; (▲) Kojic acid.

state, the molds may be damaged by shear stress while the cells in the interior of pellets are prone to undergo autolysis due to oxygen deficiency. On the other hand, the production rate of kojic acid in the surface culture is usually limited by mass transfer rate where oxygen uptake by the cells is not efficient. In order to overcome problems and disadvantageous exist in the submerged and surface cultures, Yasuhara et al. (1994) proposed a novel cultivation method termed as the membrane surface liquid culture. In this culture system, molds are grown on the surface of a microporous membrane that faces the air with its opposite side in contact with the liquid medium. The membrane surface liquid culture has both the advantageous features of surface and submerged cultures. In the membrane surface liquid culture, molds are grown on the surface of a porous membrane facing the air, similar to the surface culture, and no agitation. However, the liquid medium is used as in a conventional submerged culture, where the culture conditions can be easily controlled during fermentation.

The membrane surface liquid culture had been applied for the production of kojic acid by *A. oryzae* NRRL484 (Ogawa et al., 1995). The researchers reported that the maximum concentration of kojic acid (30 g/L) and the production rate obtained in the membrane surface liquid culture were higher than those obtained in the shaking culture (20 g/L). Since the amount of energy required for agitation and aeration is negligible and downstream processing is simple because the medium is not contaminated with cells, it can be suggested that the membrane surface liquid culture is an energy-saving process. Nonetheless, some technical problems related to scaling-up of the process such as the method of spore inoculation, removal of cells and sterilization of apparatus should be resolved before it can be used as an industrial process.

Resuspended cell system

Kojic acid production phase could be extended by the addition of glucose to the replenishment culture after growth almost ceased under high aeration rate (Kitada et al., 1967), suggesting that**,** the mycelia cells were still active in synthesizing kojic acid even after prolonged incubation. The cell-bound enzyme system that is responsible in kojic acid biosynthesis is stable for prolonged incubation in glucose solution (Bajpai et al., 1982). This special characteristic of the cell-bound enzyme has led to the development of resuspended cell system for production of kojic acid. The researchers also found that mycelia, grown in the yeast extract sucrose (YES) medium and resuspended in a buffer containing only carbohydrates, produced kojic acid almost to the same extent as in the case when a complete growth medium was used. These observations suggest that**,** limitation of nitrogen source is required to suppress the growth during resuspended cell system, so that**,** more glucose can be converted to kojic acid. The use of resuspended cell system for biotransformation of glucose to kojic acid has recently gathered a new momentum of interest in a view of its capability to produce pigment-free kojic acid, which will make the purification process much

easier. The kinetics of biotransformation of glucose and sucrose to kojic acid by resuspended cell system of *A. flavus* has recently been reported by Rosfarizan and Ariff (2007).

Immobilised cell system

Immobilised spores of *A. oryzae* in calcium alginate beads have been used as biocatalyst for kojic acid production (Kwak and Rhee, 1992). In order to maximize the metabolic activity of the immobilised *A. oryzae*, the amount of growth limiting nutrients and bead size was found to be a very important factor affecting mycelial distribution on polymeric gel beads and the overall reaction rates of immobilised cell cultures. Regardless of the size of the immobilised particles, there existed an optimal nitrogen concentration for the maximum production rate of kojic acid, at which smaller bead sizes resulted in a higher kojic acid production rate. Higher specific oxygen uptake rate in fermentation, with smaller bead size, might be one of the factors which increased the productivity, which was required to enhance the production of enzymes involved in kojic acid biosynthesis pathway (Woodhead and Walker, 1975). As mentioned earlier, high oxygen demand was required during the active growth phase of *A. flavus* for the enhancement of enzyme synthesis relevant to kojic acid production (Ariff et al. 1996).

The immobilised cells of *A. oryzae* in calcium alginate beads could also be applied in repeated batch fermentation under long-starvation conditions (Kwak and Rhee, 1992). The immobilised cells were capable of producing kojic acid linearly up to 83 g/L with fermentation time, while maintaining the stable metabolic activity for a prolonged production period (528 h). In freely suspended cells, the maximum concentration of kojic acid obtained was only 18 g/L.

CONCLUSION

Kojic acid has many industrial applications and its demand is increasing enormously with the growing industries related to its applications. Although, various fermentation techniques can be applied for kojic acid production, it was proven that, the batch submerged fermentation gives the highest efficiency in terms of yield, maximum kojic acid concentration and also overall productivities. In general, it can also be concluded that glucose and yeast extract are the preferred carbon and nitrogen sources for kojic acid production by various fungal strains.

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