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Citric acid production from carob pod by solid-state fermentation

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The production of citric acid from carob pod by Aspergillus niger *in solid-state fermentation was investigated. The maximal citric acid concentration (176 ± 4 g kg^{-1} dry pod), biomass dry weight (30 ± 0.7 g kg^{-1} wet substrate), citric acid yield (55 ± 2%), and sugar utilization (64 ± 2.5%) were obtained at a particle size of 0.5 mm, moisture level of 65%, pH of 6.5, and temperature of 30°C. The addition of 6% (w/w) methanol into the substrate increased the concentration of citric acid from 176 to 264 g kg^{-1} dry pod.*

Keywords: Citric acid; carob pod; *Aspergillus niger*; solid-state fermentation

Introduction

The carob pod is the fruit of the carob tree (*Ceratonia siliqua*) which is mainly cultivated in the Mediterranean countries and in many areas of North America. The annual production is about 340,000–400,000 metric tons. Greece is a primary producer with an annual harvest of 21,000 tons.[1] From the utilization viewpoint, two parts can be distinguished in the pod: the kibble or "locust bean" and the seeds or "locust kernel gum", a galactomannan highly valued in the food, textile, and cosmetic industries. The carob kibble contains the following expressed as g (100 g)$^{-1}$ kibble: moisture, 10–15; total sugars (glucose, fructose, sucrose, and maltose), 40–50; protein, 3–4; pectin 1–2; cellulose, 7; hemicellulose, 5; phenolic compounds, 20; fat, 0.5–1.0; and ash, 2–3.[1] Before the twentieth century, the carob pods were exclusively used as animal fodder and also for human consumption. In more recent years, most of the carob pods are still being used in animal feeds. Several applications of the kibble are in use. It is used in the preparation of antiarrheic and antiemetic products, pastry baking, and as cocoa substitution.[2] Because of the high concentration of sugars in the carob kibble, it is important to develop new and more attractive uses of these sugars.

Citric acid, a tricarboxylic acid, is used in the pharmaceutical, food, and beverage industries as an acidifying and flavor-enhancing agent. In the last years, a considerable interest has been shown in using agricultural products and their wastes such as date, maize, citrus, and kiwifruit peel, apple and grape pomace, pineapple, mandarin orange, and brewery wastes for citric acid production by *Aspergillus niger*.[3–11] The production of citric acid from carob pods by solid-state fermentation has not been investigated.

The aim of this investigation was to examine the potential of carob pods as a source for citric acid production by *A. niger* via solid-state fermentation as well as to study the effect of various fermentation parameters such as particle size, moisture, pH, temperature, and methanol concentration on kinetic parameters of carob pod fermentation.

Materials and methods

Microorganism

A. niger ATCC 9142 (American Type Culture Collection, Rockville, MA) was used throughout this investigation. It was maintained on potato dextrose agar slants at 4°C and subcultured in intervals from 1–2 months.

Inoculum

The cultures were incubated on potato dextrose agar slants at 30°C for 5 days. The spores obtained were suspended in 5 ml of sterile-distilled water to prepare the inoculum.

Fermentation medium

Carob pods (cultivar *Tylliria*) were obtained from the local market. After removing the seeds, kibble was chopped into small particles ranging from 0.3–0.6 cm and pulverized in a Waring Blender at

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high speed. The pulverized particles were dried overnight in an oven at 70°C and passed through sieves with a pore size of 0.5, 1.2, 2.5, and 5.0 mm. Carob kibble (20 g; particle size, 0.5 mm) containing 10 g initial sugars was placed in 500-ml conical flasks and moistened with the appropriate amount of distilled water in order to contain 65% moisture. The pH of the substrate was adjusted to 6.5 with 1 N NaOH. The medium was sterilized at 121°C for 30 min and inoculated with 5 ml of inoculum containing 1.0×10^8 spores ml^{-1}. The flasks were incubated at 30°C in an incubator under stationary conditions.

Study of fermentation parameters

Particle size

A set of conical flask experiments was performed at different particle sizes (0.5, 1.2, 2.5, and 5.0 mm) with 65% moisture and pH 6.5. The flasks were inoculated with 5 ml of inoculum and incubated at 30°C for 12 days.

Moisture content

A series of conical flasks containing 20 g carob kibble (particle size, 0.5 mm; pH 6.5) were moistened with an appropriate amount of distilled water in order to contain 55, 60, 65, and 70% moisture. The flasks were inoculated and incubated as above.

Initial pH

The substrate consisting of 20 g carob kibble (particle size 0.5 mm) with 65% moisture and at pH 3.5, 4.5, 5.5, or 6.5 was inoculated with 5 ml of inoculum and incubated at 30°C for 12 days.

Temperature

The medium (20 g carob kibble; particle size, 0.5 mm; moisture, 65%; and pH 6.5) was inoculated with 5 ml of inoculum and incubated at different temperatures (25, 30, 35, and 40°C) for 12 days.

Effect of methanol

A set of conical flask experiments were performed at different methanol concentrations [1.5, 3.0, 4.5, 6.0, and 7.5 g methanol $(100\ g)^{-1}$ wet substrate] to investigate the influence of methanol on kinetic parameters of citric acid fermentation. The flasks containing 20 g carob kibble (particle size, 0.5 mm; moisture, 65%; and pH 6.5) were inoculated with 5 ml of inoculum and incubated at 30°C for 12 days. Methanol was added after sterilization of the medium.

Analytical techniques

At appropriate time intervals, fermentation flasks were removed and the contents analyzed. The mycelium was removed from the flask, washed twice with 100 ml distilled water, and dried at 105°C to constant weight. The fermented mash was mixed with the above 200 ml effluent of the mycelium and the mixture was shaken on a rotary shaker/incubator (Lab-Line Orbit-Environ Shaker, Lab-Line Instruments, Inc., Melrose Park, IL) at 250 rpm for 30 min at 30°C in order to extract the citric acid and the residual sugars from the mash. The extract was then centrifuged at 4,000 *g* for 15 min and the sediment was treated again as described above with 200 ml distilled water for the complete extraction of the fermented materials. The supernatants of the two extraction treatments were mixed together and the mixture was used for the determination of citric acid and residual sugars as described previously.[12] The pH of the fermented mash was measured using a knick 646 pH meter equipped with a glass electrode. Citric acid yield was expressed as *g* citric acid $(100\ g)^{-1}$ sugar consumed. Sugar utilization was calculated by dividing the sugar consumed during fermentation by the initial sugar and multiplying the result by 100.

Each experiment was repeated three times and the results were reported as averages $\pm$ SD of three repetitions.

Results and discussion

Citric acid production via solid-state fermentation

The production of citric acid from carob kibble by *A. niger* in solid-state fermentation is shown in *Figure 1*. The concentration of citric acid increased with the increase in fermentation time. The maximum citric acid concentration (176 g kg^{-1} dry pod) was obtained after 12 days of fermentation and then declined on the 15th day. The decline in concentration of citric acid may have been due to a decay in the enzyme system responsible for the production of citric acid upon exhaustion of the fermentable sugars.[13] Roukas and Alichanidis[14] reported that a high concentration of citric acid (65 g l^{-1}) was obtained when *A. niger* was grown in beet molasses in surface fermentation. Hang et al.[6] and Hang and Woodams[24] reported maximum citric acid concentrations of 100 g kg^{-1} dry kiwifruit peel, 164 g kg^{-1} dry apple pomace, and 56 g kg^{-1} dry grape pomace for various *A. niger* strains grown in solid-state fermentation whereas Tran and Mitchell[9] found that a high concentration of citric acid (160 g kg^{-1}) was obtained when *Aspergillus foetidus* ACM 3996 was grown in pineapple waste. There are some possible reasons for these differences including the strain of organism used, chemical composition of the substrate, fermentation system, and generally, the conditions under which the fermentation takes place.

The biomass dry weight followed a pattern similar to citric acid concentration with maximum biomass concentration observed at the same time as the maximum concentration of citric acid was observed (*Figure 1*). The highest mycelial dry weight (30 g kg^{-1} wet substrate) was obtained after 12 days of fermentation and then remained constant.

The pH decreased during fermentation (*Figure 1*). This was due to the citric acid production during fermentation of sugars. The lowest value of pH was accompanied with the greatest concentration of citric acid; the pH value then increased slightly due to oxidation of citric acid by the fungus.[15]

As expected, the concentration of residual sugars decreased during the fermentation, coinciding with an increase in biomass and citric acid production (*Figure 1*). The lowest concentration of residual sugars (63 g kg^{-1} wet substrate) was observed after 12 days of incubation. At this time, 55% of sugars consumed was converted to citric acid while the total amount of utilized sugars was 64%.

Effect of particle size

The effect of particle size on kinetic parameters of carob pod fermentation is shown in *Figure 2*. As shown in *Figure 2*, the kinetic parameters decreased significantly with the increase in particle size from 0.5–5 mm. The maximal citric acid concentration (176 $\pm$ 4 g kg^{-1} dry pod), biomass concentration (30 $\pm$ 0.7 g kg^{-1} wet substrate), citric acid

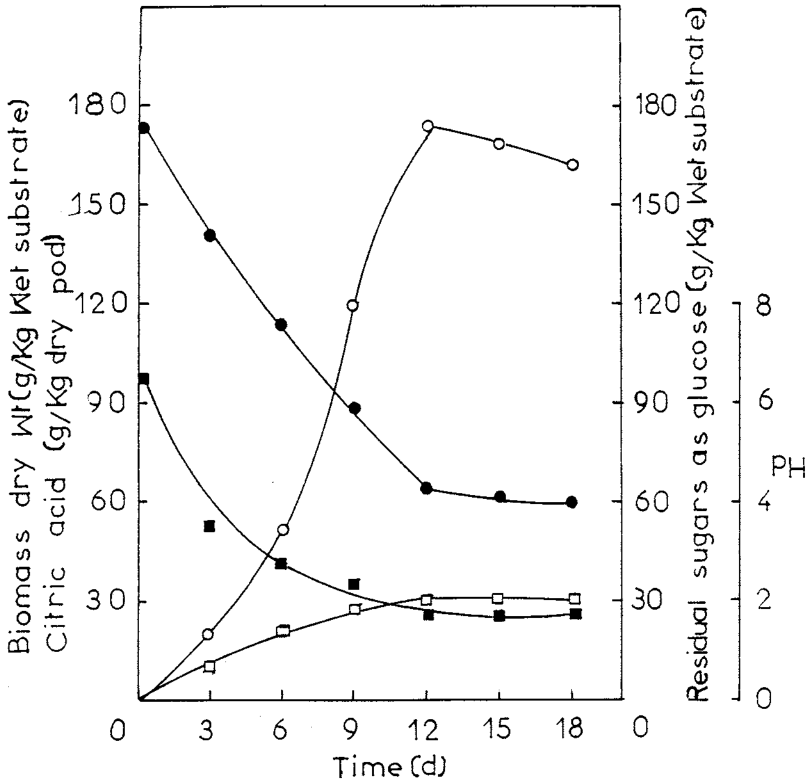

Figure 1 Fermentation kinetics of *A. niger* ATCC 9142 during citric acid production from carob pod in solid-state fermentation. Citric acid, ❍; biomass dry weight, □; residual sugars as glucose, ●; and pH, ■. Each point is the mean of three repetitions. The initial conditions were pH 6.5; particle size, 0.5 mm; moisture, 65%; and temperature, 30°C

yield (55 ± 2%), and sugars utilization (64 ± 2.5%) were obtained with the finest particle size (0.5 mm) whereas the above parameters were decreased with the coarsest particle size (5.0 mm). This indicates that in fermentations with small particles, sufficient surface area was available for adequate sugar diffusion and hence *A. niger* growth and citric acid production. On the other hand, in the case of larger particles, the reduced surface area/volume ratio provided a smaller surface for *A. niger* growth and might have inhibited penetration of microorganism cells into the carob

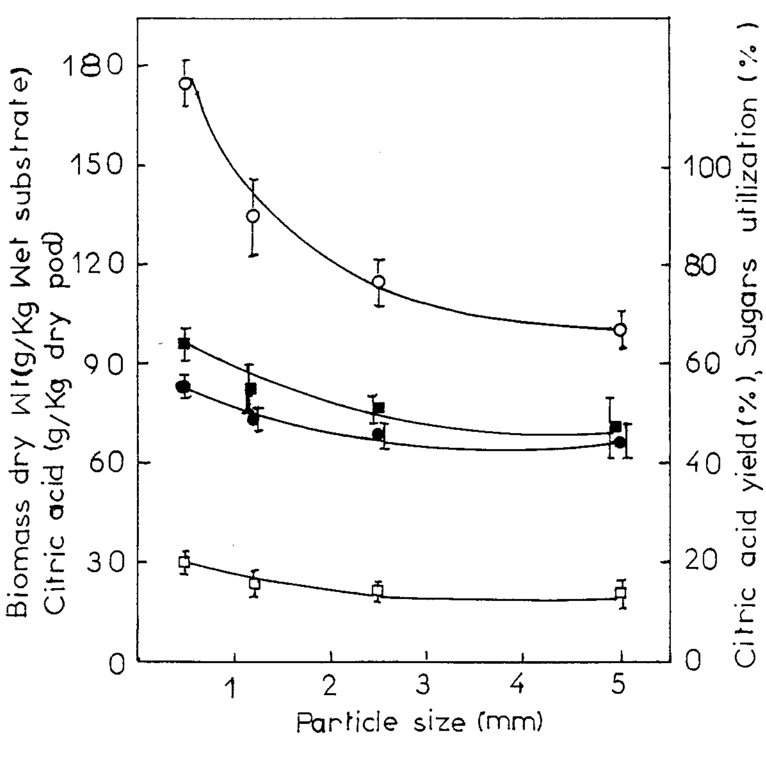

Figure 2 Kinetic parameters of carob pod fermentation by *A. niger* ATCC 9142 at different particle size. Citric acid, ❍; biomass dry weight, □; citric acid yield, ●; and sugar utilization, ■. Each point is the mean ± SD of three repetitions

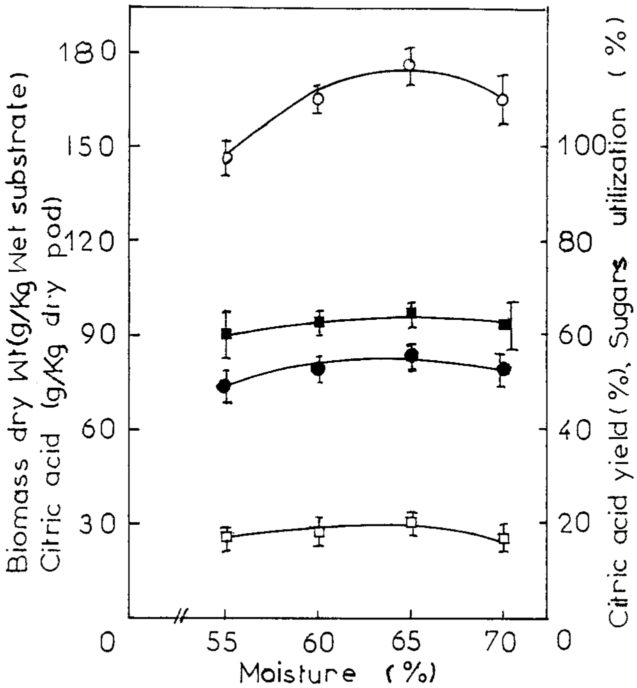

Figure 3 Kinetic parameters of carob pod fermentation by *A. niger* ATCC 9142 at different moisture levels. Symbols are the same as in *Figure 2*. Each point is the mean ± SD of three repetitions

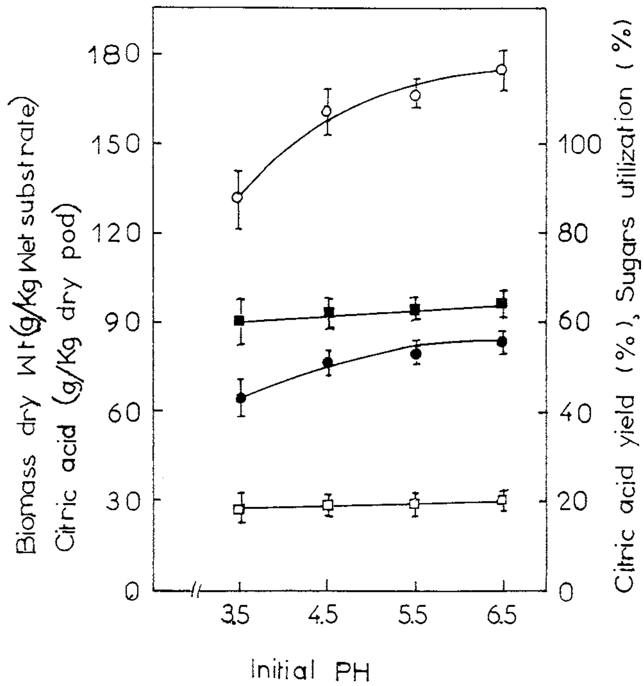

Figure 4 Kinetic parameters of carob pod fermentation by *A. niger* ATCC 9142 at different pH values. Symbols are the same as in *Figure 2*. Each point is the mean ± SD of three repetitions

kibble particles.[16] The above results agree with those of Gibbons and Westby[16] and Amin[17] who studied the effect of particle size on ethanol production from fodder beet and sugar beet, respectively. The results showed that the maximum citric acid concentration was obtained with the finest particle size; however, the energy consumption for grinding to tiny particles is high,[18] so the amount of citric acid produced should be correlated with the grinding cost.

Effect of moisture content

One important factor that affects the performance of solid-state fermentation is the moisture content of solids. The purpose of this experiment was to determine the optimum moisture level of carob kibble that would result in the highest citric acid concentration. As shown in *Figure 3*, the citric acid concentration, citric acid yield, biomass dry weight, and sugar utilization were increased with the increase in moisture content. The highest values of fermentation parameters were achieved at a moisture level of 65%. Tran and Mitchell[9] reported that a maximum citric acid concentration was obtained from pineapple waste at a moisture level of 70% when *A. foetidus* ACM 3996 was grown in solid-state fermentation. Decreasing the moisture level from 65 to 55% resulted in a decrease in the kinetic parameters. The decrease in moisture level is advantageous since the chance of contamination in the fermentation medium is reduced; however, there is a lower limit of moisture content below which *A. niger* may not function to produce citric acid. This may be due to the higher osmotic pressure levels at lower moisture contents.[19] Ngadi and Correia[20] reported that low substrate moistures in solid-state fermentation resulted in suboptimal product formation due to reduced mass transfer processes such as diffusion of solutes and gas to the cell during fermentation.

Effect of initial pH

An important factor that affects the performance of carob pod fermentation is the initial pH of the substrate. The purpose of this experiment was to determine the optimum initial pH of carob pod that would result in the highest citric acid concentration. As shown in *Figure 4*, the citric acid concentration and the citric acid yield increased with the increase in initial pH from 3.5–6.5. On the other hand, the biomass dry weight and the sugar utilization remained almost constant over the pH range 3.5–6.5. The highest values of the above fermentation parameters were achieved at an initial pH of 6.5. These results agree with those of Roukas and Alichanidis[21] who studied the effect of initial pH on citric acid production from beet molasses by surface fermentation.

Effect of temperature

The effect of temperature on kinetic parameters of carob pod fermentation is shown in *Figure 5*. The citric acid concentration increased significantly with the increase in fermentation temperature from 25–30°C and decreased above 30°C. This was due to the denaturation of the enzyme system of microorganism at high temperatures.[22] The biomass dry weight and the sugar utilization increased slightly with the increase in fermentation temperature from 25–40°C. Szewczyk and Myszka[23] studied the effect of tem-

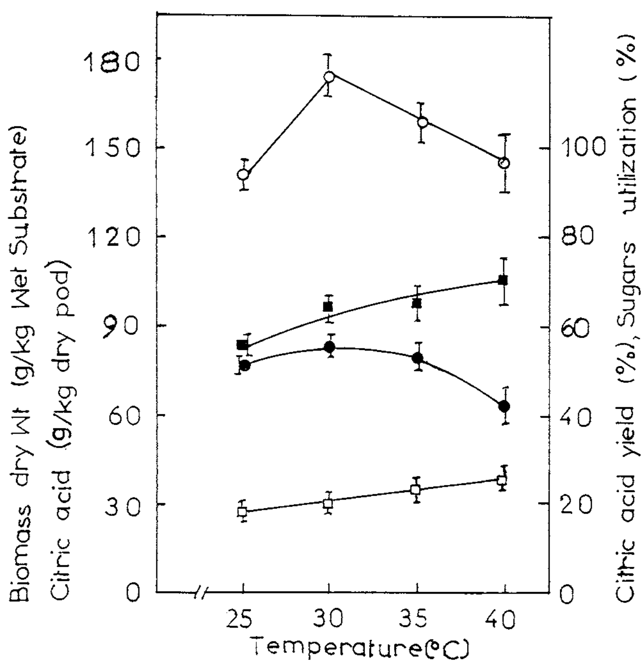

Figure 5 Kinetic parameters of carob pod fermentation by *A. niger* ATCC 9142 at different temperatures. Symbols are the same as in *Figure 2*. Each point is the mean ± SD of three repetitions

perature on *A. niger* growth in solid-state fermentation found that the temperature did not strongly affect the growth rate in the range of 28–34°C. No significant differences were noted in citric acid yield among cultures grown at 25, 30, and 35°C. The maximum citric acid concentration (176 ± 4 g kg^{-1} dry pod) and citric acid yield (55 ± 2%) were obtained in culture grown at 30°C while the biomass dry weight and the sugar utilization were maximum at 40°C. These results agree with those of Hang and Woodams[24] who studied the effect of temperature on citric acid production from grape pomace by solid-state fermentation.

Effect of methanol

As shown in *Figure 6*, the citric acid concentration and the sugar utilization increased with the increase in methanol concentration from 1.5–6% (w/w) and decreased as the methanol concentration was increased beyond 6%. On the other hand, the biomass dry weight and the citric acid yield remained almost constant with the increase in methanol concentration from 1.5–6%. The highest values of citric acid concentration (264 ± 12 g kg^{-1} dry pod), biomass dry weight (32 ± 5 g kg^{-1} wet substrate), citric acid yield (60 ± 4%), and sugar utilization (87 ± 3%) obtained in the presence of methanol at a concentration of 6% (w/w). Hang et al.[25] and Roukas and Kotzekidou[26] reported that the addition of methanol at concentrations of 1–4% (v/v) resulted in a marked increase in the amount of citric acid formed by *A. niger* on spent grain liquor and brewery wastes, respectively. The observed increases in citric acid concentration show that methanol has a profound effect on the metabolism of sugars by *A. niger*. The mechanism by which methanol stimulates citric acid production from sugars is not clear. Maddox et al.[27] reported that the effect of methanol is at the cell permeability level, allowing citrate to be excreted from the cell; the cell then responds by increasing its citrate production via repression of 2-oxoglu-

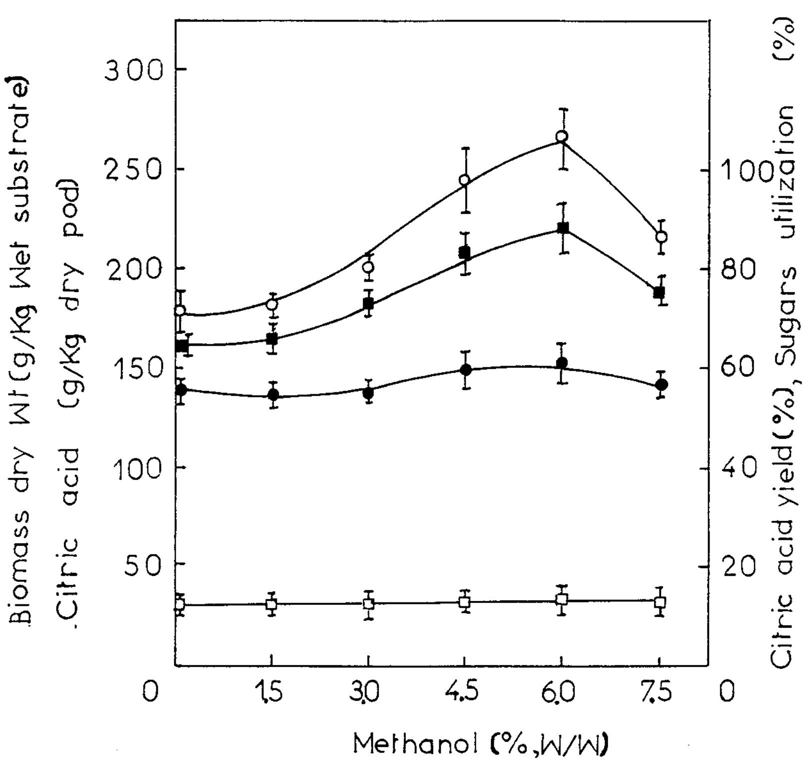

Figure 6 Effect of methanol addition on kinetic parameters of carob pod fermentation by *A. niger* ATCC 9142 in solid-state fermentation. Symbols are the same as in *Figure 2*. Each point is the mean ± SD of three repetitions

tarate dehydrogenase in an attempt to maintain an adequate intracellular level of the metabolite.

Conclusions

The results showed some important aspects of citric acid production from carob pod by *A. niger* in solid-state fermentation. The optimum conditions for carob pod fermentation were particle size, 0.5 mm; moisture, 65%; initial pH, 6.5; and temperature, 30°C. The addition of methanol at concentrations up to 6% (w/w) resulted in a marked increase in the citric acid concentration. The carob pod was an attractive medium for the production of citric acid by *A. niger*.

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