Submerged Citric Acid Fermentation of Blackstrap Molasses

Part I. Isolation of a Producing Strain Utilizing Blackstrap Molasses and the Effects of Incubation Temperature

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Isolation and screening tests were carried out in order to find microorganisms which were able to produce citric acid directly from blackstrap molasses. Some strains were obtained which accumulate considerable quantities of citric acid. Certain temperature changes during the course of incubation were found to increase the yield of citric acid.

The present investigation was undertaken to see if a simple method could be found to improve the yield of citric acid from blackstrap molasses, and we could obtain the yield of more than 70% from the untreated molasses using a newly isolated strain of *Asp. niger*.

INTRODUCTION

It has been known that the production of citric acid by fermentation is greatly affected by the metal ions and other components in the medium.¹⁻³ These factors make citric acid more difficult and cause many problems in industrial production. One step towards providing suitable conditions for citric acid accumulation is to decrease the metal concentrations well below those for optimal growth.⁴⁻⁶ Another is to add a chemical agent such as methanol.^{7,8} There are, however, many uncontrollable factors, especially in the use of crude carbohydrates.

In the production of citric acid, an organism is desired which can utilize high concentrations of relatively crude carbohydrates

rapidly. This probably means either an induced mutant with extremely high metal requirements, or an organism with a defective enzyme system which can accumulate citric acid even in a complete medium.

In view of the industrial importance of producing citric acid by the submerged fermentation of low cost carbohydrates, with screening for high citric acid-producing strain which are tolerant of various uncontrollable factors was performed. Further work was undertaken to develop a simple method for increasing the citric acid yields. Certain temperature changes in the fermentation after twenty-four hours was observed to be effective.

EXPERIMENTAL AND RESULTS

I. Isolation of Strains Capable of Producing High Citric Acid Titres from Blackstrap Molasses

Microorganisms were isolated from a variety of sources including soils leaves and dead fruits. About 1 g. of sample was suspended in 10 ml of sterilized water and the suspension was plated on the agar medium composed

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as follows: 250 g. blackstrap molasses, 0.05 g. NH_4NO_3 , 0.5 g. KH_2PO_4 , 0.5 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 30 g. agar powder and water to 1,000 ml and pH was adjusted to 3.5 by dilute HCl. Unless the pH is lowered to 3.5, unwanted microorganisms will grow abundantly. Below pH 3.5, the medium will not harden. After 48 hours incubation at 30°C, the organisms grown on the agar plates were transferred to the blackstrap molasses agar slant which is composed of the same medium. Philippine blackstrap molasses was used throughout the experiments, and was supplied by Sanraku Ocean Co., Ltd. Analytical data of this molasses is shown in Table IV and its content of total nitrogen, Fe, P and Ash were 0.54, 0.045, 0.125 and 6.85% respectively. To find a suitable medium and optimum conditions of screening, the preliminary experiments varying pH, and temperature were carried out using a newly isolated strain, *Asp. niger* N-9.

Blackstrap molasses agar slants of the sporulation medium were inoculated with *Asp. niger* N-9 and incubated at 30°C for 5 days. Then 10 ml of sterile distilled water was poured over the surface growth and stirred with a sterile needle to detach and separate the spores. Suspension of spores

of this strain in distilled water was prepared by filtering through the sterile cotton cloth. Each 200 ml Erlenmeyer flask was charged with 50 ml of medium. Cultivation was performed on a rotary shaker at 150 r.p.m., for seven days. This strain was confirmed no other acid than citric acid was produced by paperchromatography.

TABLE II. RESULT OF THE PRELIMINARY TEST

Medium no.	Starting pH	Temperature °C	Acidity ml/100 ml
1	5.6	26.5	27.5
1	5.6	30.0	23.6
1	3.0	26.5	26.3
1	3.0	30.0	24.8
2	5.6	26.5	28.1
2	5.6	30.0	23.3
2	3.0	26.5	21.3
2	3.0	30.0	18.8
3	5.6	26.5	16.0
3	5.6	30.0	14.3
3	3.0	26.5	19.7
3	3.0	30.0	13.9
4	5.6	26.5	7.6
4	5.6	30.0	6.4
4	3.0	26.5	11.0
4	3.0	30.0	6.1
7	5.6	26.5	28.8
7	5.6	30.0	28.5
7	3.0	26.5	27.4
7	3.0	30.0	24.3
8	5.6	26.5	29.3
8	5.6	30.0	28.2
8	3.0	26.5	25.2
8	3.0	30.0	26.3
9	5.6	26.5	28.0
9	5.6	30.0	27.3
9	3.0	26.5	26.0
9	3.0	30.0	27.6
10	5.6	26.5	29.0
10	5.6	30.0	28.0
10	3.0	26.5	26.5
10	3.0	30.0	25.4
11	5.6	26.5	5.8
11	5.6	30.0	3.8
11	3.0	26.5	8.5
11	3.0	30.0	3.6

(Culture for 7 days on a rotary shaker, sugar content ca. 13%)

TABLE I. CULTURE MEDIUM EMPLOYED FOR PRELIMINARY TEST

Medium No.	Molasses g/100 ml	NH_4NO_3 g/100 ml	KH_2PO_4 g/100 ml	MgSO_4 g/100 ml
1	25	—	—	—
2	25	0.05	—	—
3	25	0.10	—	—
4	25	0.15	—	—
5	25	0.20	—	—
6	25	—	0.03	—
7	25	—	0.10	—
8	25	—	0.03	0.03
9	25	—	0.10	0.10
10	25	—	—	0.03
11	25	0.20	0.03	0.03

The titratable acidity was determined by titration of 5 ml aliquots of the culture medium with 0.10 N sodium hydroxide. One milliliter of this alkali is equivalent to 6.4 mg of anhydrous citric acid. Residual sugar was determined as glucose by the method of Shaffer and Somogyi.⁹⁾ Citric acid was analysed by the method of Natelson et al.^{10,11)} In most cases the percentage conversion of citric acid from the sugar consumed was calculated from the titratable acidity and routinely the citric acid was determined by paper chromatography. The standard organic acids solutions were developed on the paper to identify the spots.

Table I shows the culture medium for the preliminary test and the Table II shows the results of this experiments.

Monopotassium phosphate and magnesium sulfate little affected the acid yield. As to initial pH, 5.6 appeared optimum for acid yield. The optimum temperature was found to be 26.5°C. Increased amount of added nitrogen gave a decrease in citric acid yield.

In view of the above facts, the most reasonable conclusion to be drawn was that cultural conditions for screening should be set to include dilution of the molasses, pH 5.6 and temperature 26.5°C.

Screening test was done by the above condition and the results of it were summarized on Table III.

As it is evident from the Table III, high citric acid-producing strains are widely distributed among the nutritionally rich cultivated fields, and are rarely existent in the nutritionally poor air, roads, grounds, forest soils etc. It is not clear the relation between

TABLE III. DISTRIBUTION OF ACID-PRODUCING STRAINS

Collecting sources	Samples	Select-ed strains	Acid Maxi-mum	product-ivity Mini-mum	Mean acidity	Strains, more than 20 ml.
Field, Cultivated farm soil	65	191	24.6	5.1	7.5	11
Meadow	45	65	8.0	3.7	6.1	0
Forest Mountainous district	18	47	13.3	4.1	8.8	0
Dead leaf	27	29	10.5	2.0	7.4	0
Dead fruit	37	36	13.0	1.2	7.4	0
Road mud	25	40	6.8	1.7	4.2	0
Air	22	18	9.5	3.5	7.5	0
Mine	10	17	13.7	4.2	8.5	0
the rest	269	443	—	—	7.44	11

citric acid producing strains and habitat. However, it may be considered that there is some relation between citric acid productivity of strain¹²⁾ and sand culture which is considered an effective method to maintain the productivity of citric acid.

Contrary to the expectation, the high citric acid producing strains were not isolated from low pH sources such as greens and fruits. Results of this may be said that the pH of medium is little importance in the screening for high citric acid-producing strains.

Out of the 443 strains isolated, 11 strains were found which produced considerable citric acid from 25% blackstrap molasses. Three of them, N-9, N-88 and N-227, were the most promising. The conversion was between 45~55% per sugar consumed by shaking culture.

II. Comparable Test of Different Blackstrap Molasses

To assure the high citric acid productivity on any black-strap molasses of the isolated strain, *Asp. niger* N-88, comparable test of different blackstrap molasses were performed.

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TABLE IV. COMPONENT OF THE BLACKSTRAP MOLASSES

	Phillippine	Java	Burma	Australia
Total sugar	56.8(%)	53.8(%)	56.4(%)	48.0(%)
Direct sugar	21.4	20.1	26.3	20.4
Total nitrogen	0.54	0.41	0.31	0.86
Crude ash	6.85	8.04	5.00	8.79
pH	5.5	5.8	5.6	5.4
Water content	24.8	23.3	26.2	25.2

TABLE V. RESULT OF THE ACID PRODUCTIVITY BY DIFFERENT BLAKSTRAP MOLASSES

	Phillippine	Java	Burma	Australia
Acidity	56.20	46.40	36.60	17.00
Residual sugar g/100 ml	1.31	4.30	5.52	1.25
Consumed sugar g/100 ml	11.54	9.00	8.58	10.45
Acid production rate per consumed sugar (%)	63.40	67.00	55.20	21.20

(Cultured on a rotary shaker at 26.5°C for 6 days.)

Components of the blackstrap molasses of four different kinds are shown on Table IV.

Citric acid productivity tests were carried out using the above blackstrap molasses and the results are shown in Table V. As shown in Table V, excepting the Australian molasses, each of them showed considerable yields. In components, large differences of them are nitrogen, ash and total sugar contents. Australian molasses resulted in poorer acid yields and compared with others, shows that nitrogen is considerably rich, 30~40% more than others. This will be expected the preliminary test that rich nitrogen is not preferable to acid productivity. Other components of the molasses seemed not to be affected appreciably by this strain.

III. Effect of Temperature and Inoculum Size

R. Steel et al.¹²⁾ and others reported that the inoculum size has a considerable effect on the production of citric acid in submerged fermentation. A study was made of the effect

of the temperature and the inoculum size on the production of citric acid using the strain *Asp. niger* N-88. Unless otherwise stated, N-88 was used through the experiments. Preparation of spores and the culture method were same as mentioned above. Dry weight of Mycelia was thus determined by filtration of the fermenting medium, washing twice with physiological sodium chloride solution and finally by distilled water. It was then dried in the vacuum sulfuric acid desiccator for one or two days.

The experiments were carried out at 10⁴, 10⁵ and 10⁶ spores per culture of milliliter on inoculum size and temperature at 26.5°, 30° and 37°C level.

a. Effects of temperature and inoculum size on mycelial growth

Table VI shows the mycelial weight at 26.5°C was some 30 per cent lower than at 30° and 37°C and there was no significant difference between 30° and 37°C. Little difference was seen between inoculum sizes at the same temperature.

TABLE VI. EFFECT OF TEMPERATURE AND INOCULUM SIZE ON MYCELIAL GROWTH (W)*

Temperature	26.5°	30°	37°	Mean
Inoculum size				
10 ⁴ **	2.322	3.103	3.432	2.952
10 ⁵	2.576	3.615	3.706	3.299
10 ⁶	2.572	3.359	3.536	3.156
Mean	2.490	3.359	3.558	—

* Inoculum size; spores per ml.

** Mycelial weight; g/100 ml.

TABLE VII. EFFECT OF TEMPERATURE AND INOCULUM SIZE ON SUGAR CONSUMPTION (S)*

Temperature	26.5°	30°	37°	Mean
Inoculum size				
10 ⁴ **	8.88	9.56	10.13	9.52
10 ⁵	9.02	10.11	8.99	9.37
10 ⁶	9.03	9.92	10.25	9.73
Mean	8.98	9.86	9.79	—

* Sugar consumption; g/100 ml.

** Inoculum size; spores per ml.

b. Effects of temperature and inoculum size on sugar consumption

At 26.5°C sugar consumption was about 10 per cent lower than that of at temperature 30° and 37°C. Inoculum size had any significant effect on sugar consumption (Table VII).

c. Effects of temperature and inoculum size on acid yield

As to acid yield per consumed sugar, the result showed about 50 per cent at 26.5°C but at 30° and 37°C the yield was reduced by one half. Inoculum size had no any significant effect on acid yield in this experiment (Table VIII).

d. Table IX shows the acid productivity per mycelial weight. At 26.5°C, it was 3~5 times higher than that of at temperature 30° and 37°C.

e. In reference to sugar consumption per mycelial weight, there were little distinguished difference among 26.5°C, 30°C and 37°C as shown on Table X.

Based on these results it may be considered

TABLE VIII. EFFECT OF TEMPERATURE AND INOCULUM SIZE ON ACID YIELD (A/S)*

Temperature Inoculum size	26.5°	30°	37°	Mean
10 ⁴	51.70	42.65	16.50	36.20
10 ⁵	48.30	36.25	12.95	32.50
10 ⁶	50.29	35.70	14.00	33.33
Mean	50.10	38.20	12.48	—

* Yield indicates citric acid g/consumed sugar g %

TABLE IX. EFFECT OF TEMPERATURE AND INOCULUM SIZE ON ACID PRODUCTIVITY OF MYCELIA (A/W)*

Temperature Inoculum size	26.5°	30°	37°	Mean
10 ⁴	19.89	8.86	5.15	11.23
10 ⁵	16.74	9.74	3.24	9.80
10 ⁶	19.05	6.26	3.67	9.65
Mean	18.56	8.23	4.02	—

* Acidity ml/mycelial weight

that sugar consumption velocity and acid productivity have not a simple relationship. The acid productivity of mycelia is very sensitive to temperature and is not be dependent simply on mycelial weight.

TABLE X. EFFECT OF TEMPERATURE AND INOCULUM SIZE ON SUGAR CONSUMPTION PER MYCELIAL WEIGHT (S/W)*

Temperature Inoculum size	26.5°	30°	37°	Mean
10 ⁴	5.17	4.23	4.19	4.53
10 ⁵	4.69	3.94	3.29	3.97
10 ⁶	4.74	4.12	3.84	4.23
Mean	4.87	4.10	3.77	—

* Sugar consumption g/mycelial weight g

IV. Effect of Temperature Change during Inoculation

From the previous experiments it has been known that the temperature of the fermentation is a very important factor in citric acid production and that the effect of temperature change in the fermentation course have been studied.

Incubation temperatures are 22°, 24°, 26.5°, 30° and 37°C and temperature changed have been made at intervals of 24, 48 and 42 hours. The optimum temperature of the culture is different for growth and for acid forming phases. Fig. 1 shows the relation between acid productivity and culture temperature in the fermentation. The optimum temperature for

FIG. 1. Relation between Temperature of Incubation and Citric Acid Productivity

FIG. 2. Effect of Temperature Change on Citric Acid Yield in the Course of Incubation

A: 37~22 B: 37~24 C: 30~22
D: 30~24 E: 26.5~24 F: 26.5~22

FIG. 3. Effect of Temperature Change on Sugar Consumption Rate per Mycelial Weight (S/W).

A: 37~22 B: 30~22 C: 26.5~22

FIG. 4. Effect of Temperature Change on Acid Productivity.

A: 37~22 B: 30~22 C: 26.5~22

acid production is considered to be 22°C for strain *Asp. niger* N-88.

The effect of temperature changes at intervals of 24 hours, 48 hours, and 72 hours are shown on Fig. 2. Temperature change after

incubation for 24 hours resulted a high acid yield, especially when the fermentation was incubated at 37°C for 24 hours, and then transferred to 22°C for the remainder of the fermentation. The bigger the difference of the temperature changes, the larger the effect on the acid yield. In a case of temperature drop from 30°C had little effect in acid yield.

Effect of temperature change on sugar consumption and acid productivity are shown on Fig. 3 and Fig. 4. They indicate that the temperature change at 24 hours incubation were effective. Germination phase and growth phase are at the same time enzyme forming phases and the temperature change will be effective on its formation for citric acid accumulation.¹²⁾

V. Effect of Methanol Addition on Citric Acid Fermentation

It has been found that the use of the low molecular weight alcohols, such as methanol, ethanol or propanol as adjuncts to the culture medium greatly increased citric acid production in both surface and submerged culture. The use of such alcohols has generally been attributed to the increased tolerant levels of harmful trace elements. It has been in the previous section that the temperature change

FIG. 5. Effect of the Time of Methanol Treatment on Citric Production.

○—○ Methanol added to the medium at respective time.
● Methanol added from the beginning.
×—× Mycelia were replaced to the Methanol none medium at respective time.

at 24 hours of incubation increased considerably the productivity of citric acid and the same technique was used to the addition of methanol.

A study has been made of the relation between the time of methanol addition and the citric acid production. Cultivation was performed on a rotary shaker at 26.5°C. The treatment conditions were shown on Table XI and results were shown on Table XII and Fig. 5.

As seen from Table XII and Fig. 5, methanol addition has apparently its efficiency on the citric acid production and the effect of

addition has correlation with its treating phase; addition after 24 hours incubation has the best results in acid production.

Three strains of N-9, N-88 and N-227 were isolated which were able to produce citric acid more than 50% from untreated blackstrap molasses by shaking culture. Further investigation was made to improve the acid productivity.

The change of temperature and methanol addition were effective treatments on citric acid fermentation in earlier time and these treatment gave considerable difference by treatment time on the effect.

It was found that the inoculum size of spores has little effect on citric acid yield and citric production depends largely upon the cultural temperature by these experiments. Comparing the yields culture at the temperature of 26.5°, 30° and 37°C through the fermentation, the highest yield was obtained at 26.5°C.

Experiments were carried out to determine the time effects of temperature change on citric acid production after inoculation. It was found that the culture started at higher temperature should drop to lower temperature in 24 hours after inoculation. Temperature change at 48 hours or 72 hours after inoculation resulted in lowered acid yield. From the experiments it was realized that there are optimum temperatures for mycelium producing phase and for citric acid producing phase respectively. It was shown that 30 per cent more citric acid was produced by fermentation which were started at 37°C and transferred to 22°C than was cultured at 26.5°C throughout the fermentation. There was the same tendency for the methanol addition and temperature change in citric fermentation. Methanol addition should also

TABLE XI METHANOL TREATMENT

- a: No methanol added
- b: 3 per cent of methanol was added to the medium after 24 hours incubation
- c: 3 per cent of methanol was added to the medium after 48 hours incubation
- d: 3 per cent of methanol was added to the medium after 72 hours incubation
- e: After 24 hours, 3 per cent methanol added medium was replaced to the medium free from methanol
- f: After 48 hours, 3 per cent methanol added medium was replaced to the medium free from methanol
- g: After 72 hours, 3 per cent methanol added medium was replaced to the medium free from methanol
- h: 3 per cent of methanol was added to the medium throughout the fermentation

TABLE XII. EFFECT OF METHANOL TREATMENT ON ACID YIELD

Treated condition	Citric acid yield %	Residual sugar %	Acid productivity per mycelial weight A/W	Sugar consumption per mycelial weight S/W
a	45.90	0.83	0.87	2.75
b	76.50	1.64	1.98	3.07
c	49.35	1.20	1.13	3.49
d	50.95	0.84	1.21	3.52
e	49.45	0.85	1.17	3.98
f	34.90	0.79	0.48	3.82
g	38.15	0.79	0.62	3.68
h	69.50	1.85	1.82	3.42

be made in 24 hours after inoculation or deprived of it for 24 hours.

It was shown that methanol added culture after 24 hours, produced two times of citric acid than transferred it to untreated molasses medium. Results of these may be considered the changes of cultural conditions in earlier time stimulate the mycelia preferable to produce citric acid and they will have something concerning the activity of enzymes.¹³⁾ Besides the factors discussed above resulting reasonable effect on citric acid yield, morphological discussion will be made in other paper. Inhibitory effect of methanol on mold growth will be understood by the temperature drop

in fermentation. The purpose of this paper was not to clarify the mechanism of promoting the yield of citric acid but to see if a simple method such as change of temperature could be applied to citric acid fermentation. Methanol addition or ferrocyanide treatment combined with the change of cultural temperature failed to get more desirable results.

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