# YIELD OF FUMARIC ACID FROM DIFFERENT CARBOHYDRATES BY STATIONARY SURFACE CULTURE METHOD

KM. MAYA SAIJWANI AND P. N. AGGARWAL

Defence Research Laboratory (Materials), Kanpur

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Studies on the yield of fumaric acid by *Rhizopus arrhizus* and *Bhizopus nigricans* using glucose, sucrose, xylose, cane molassess and bagasse hydrolysate either alone or in combination with glucose were undertaken. Thirty three and 26 per cent yield of fumaric acid were obtained from 12 and 10 per cent glucose media respectively, in 15 days at 33°C by stationary surface culture method. Higher yields of fumaric acid were obtained from glucose using *R. arrhizus*. Ability of this organism to produce fumaric acid from media containing (i) 50 : 50 glucose and deionized bagasse, hydrolysate (ii) deionized bagasse hydrolysate, and (iii) clarified cane molasses was investigated. A 7 per cent yield of fumaric acid was obtained from 50 : 50 glucose and deionized bagasse hydrolysate medium. In the case of xylose good growth of the fungus was obtained but no fumaric acid was produced. These studies have indicated that *R. arrhizus* is not able to utilize pentose sugars.

Waksman[1] patented a process for producing fumaric acid from various carbohydrates and carbohydrate containing materials, such as, monosaccharies, molasses, and starch by the use of selected fungi of the order Mucorales. Glucose, fructose, invert sugar, sucrose, maltose, molasses, sips, starches etc have been reported[2] to produce fumaric acid both by stationary surface culture method or by submerged culture method.

No studies appear to have been undertaken using substrates containing pentose sugars for production of fumaric acid. Hence studies were taken up to explore the possibility of using bagasse hydrolysate for production of fumaric acid. This paper describes the results of study on yield of fumaric acid obtainable from different carbohydrates/ carbohydrates containing materials including bagasse hydrolysate by stationary surface culture method.

## MATERIALS AND METHODS

### (a) *Culture and its revival*

The culture of *Rhizopus nigricans* was obtained from National Chemical Laboratory, Poona and *Rhizopus arrhizus* from Northern Regional Research Laboratory, Peoria, Illinois, USA. The freeze-dried cultures were revived in nutrient broth medium consisting of peptone 1 per cent, sodium chloride 0·5 per cent and beef extract 1 per cent. A dry pellet was dissolved in 1 ml of nutrient broth and then immediately was streaked on the nutrient agar medium consisting of beef extract 0·15 per cent, peptone 0·25 per cent, sodium chloride 0·25 per cent and agar-agar 2 per cent. Within a few days we get single colonies. These colonies were cultured on the slant medium consisting of peeled potatoes 20 per cent, dextrose 2 per cent, agar-agar 2·5 per cent for 4 days in case of *R. nigricans*,

(*b*) The sporulation medium for *R arrhizus* NRRL 2582 is as follows

| | |
|---|---|
| Commercial glucose (deionised bagasse hydrolysate or clarified molasses) | 0·4% |
| Crude lactose | 0·6% |
| Glycerol | 1% |
| Urea | 0·06% |
| Peptone | 0·16% |
| Corn Steep liquor | 0·1% |
| $KH_2PO_4$ | 0·04% |
| $MgSo_4 \cdot 7H_2O$ | 0·05% |
| $ZnSo_4 \cdot 7H_2O$ | 0·009% |
| $FeSo_4 \cdot 7H_2O$ | 0·025% |
| pH | 6·4 |
| Temp | 33°C |
| Incubation period | 4 days |

(*c*) *Preparation of deionized bagasse hydrolysate*

(*i*) Dried bagasse was cut to small bits and subjected to acid hydrolysis by refluxing with 7 per cent sulphuric acid (AR) for 8 hrs., keeping the ratio of liquor to bagasse 10 : 1. The excess of this acid was neutralized by calcium carbonate, the reducing sugars formed were 8 per cent calculated as glucose and 8·4 per cent calculated as xylose on the weight of bagasse.

(*ii*) *Analysis of bagasse hydrolysate*—The hydrolysate was found to have the following composition reported earlier by Agarwal[3] *et al* Xylose 70 per cent, glucose 14 per cent, arabinose 10 per cent and unidentified sugar 6 per cent.

(*iii*) *Deionization of bagasse hydrolysate*—Cation exchange resin- Amberlite (IRA 120 (H) ) and anion exchange resin Amberlite (IR-400 (C1) ) were prepared in convenient batches, sufficient for a number of columns, by refluxing with aqueous 10 N ammonium hydroxide and *2N* hydrochloric acid. The regenerating cation resin was kept in the acid form, anion resin in the *Cl* form.

Bagasse hydrolysate was first passed through cation exchange resin and then through anion exchange resin. After that deionised bagasse hydrolysate was concentrated under vacuum to have 12 per cent reducing sugars in the hydrolysate.

(*d*) *Clarification of molasses*

Cane molasses (pH–5·8) was obtained from the market and was clarified as described earlier by Agarwal *et al*[3].

(*e*) *Inoculum medium for R arrhizus*

| | |
|---|---|
| Commercial glucose (deionised bagasse or clarified molasses) | 1·5% |
| High test molasses | 1% |
| Urea | 0·1% |
| Corn Steep liquor | 0·3% |
| $KH_2PO_4$ | 0·03% |

| | |
|---|---|
| $MgSO_4 \cdot 7H_2O$ | 0·025% |
| $ZnSO_4 \cdot 7H_2O$ | 0·0066% |
| Ferric tartarate | 0·001% |
| Corn-starch | 3% |
| pH | 6·4 |
| Temp. | 33°C |
| Incubation period | 24 hrs. |

(*f*) *Fermentation medium for R nigricans*

| | |
|---|---|
| Glucose | 10% |
| Ammonium sulphate | 0·2% |
| $KH_2PO_4$ | 0·05% |
| $MgSO_4 \cdot 7H_2O$ | 0·05% |
| $ZnSO_4 \cdot 7H_2O$ | 0·01mg/100ml |
| Ferric sulphate | 0·02mg/100ml |
| pH | 6·4 |
| Temp. | 33°C |
| Incubation period | 15 days |

(*g*) *Fermentation medium for R. arrhizus NRRL 2582*

| | |
|---|---|
| Commercial glucose (deionised bagasse hydrolysate or clarified molasses). | 12% |
| Urea | 0·1% |
| Corn Steep liquor | 0·05% |
| $KH_2PO_4$ | 0·03% |
| $MgSO_4 \cdot 7H_2O$ | 0·04% |
| $ZnSO_4 \cdot 7H_2O$ | 0·0044% |
| Ferric tartarate | 0·001% |
| pH | 6·4 |
| Temp. | 33°C |
| Incubation period | 15 days |

(*h*) *Preparation of spore suspension*

Both fungi were grown on slant medium for 4 days at 33°C to obtain a massive crop of spores. The spores were washed with 30 ml of detergent solution (0·9 per cent NaCl solution). Filtered through sterilized muslin in sterilized inoculation chamber.

(*i*) *Fermentation*

In case of *R nigricans* 120 ml fermentation medium (composition given in para (*d*) above) was taken in a Kolle flask. The medium was sterilized, cooled and then inoculated at 33°C with the spore suspension 3 to 5 per cent and then incubated for 15 days at 33°C.

In the case of *R arrhizus* the inoculum was grown by adding 10 ml of the spore suspension to 100 ml of the inoculum medium (described at para (*e*) above) and incubation on a rotary shaker for 24 hrs. at 33°C. Three to five ml of this inoculum containing germinated spores were used for 100 ml of the fermentation medium (composition given at para (*e*) above). The inoculated Kolle flasks containing fermentation medium were taken, incubated at 33°C for 15 days and the fermentation was allowed to proceed by stationary surface culture method. After 48 hrs. of incubation, the pH of the solution became 2 while initial pH was 6·4. At this time sterilized slurry of calcium carbonate (50 per cent of the carbohydrate concentration in the fermentation medium) was added to neutralize the acid produced and the pH was adjusted to 4. When excess of $CaCO_3$ was added (pH-above 4·0), there was a Sharp reduction in fumarate formation, and Calcium fumarate formation occurred at the lower surface of the mycelial mat or at the bottom of the Kolle flasks. The crystallized calcium fumarate became so abundant that practically the entire fermentation liquid set in the form of stiff jel due to the hydrophillic nature of the salt formed.

(*j*) *Recovery of fumaric acid*

Culture filterate and the mycelial mat were separated by filteration under reduced pressure and Conc. *HCl* was added to bring the *pH 3·5*. All the residual $CaCO_3$ was thus neutralized. The mycelium was boiled in acidic water to separate out the crystals of calcium fumarate which settle down on the lower surface of the mycelium. In the culture filterate and boiled water (mycelium washed water) containing calcium fumarate (1·56 per cent soluble in water at 25°C), more *HCL* was added to bring the *pH* to 2 (at this *pH* calcium fumarate hydrolysed completely). This resulted in the heavy precipitation of finely divided fumaric acid crystals which continued on cooling. This was filtered under reduced pressure and the crystalline fumaric acid so obtained, was washed two or three times with distilled water to obtain the pure crystalline product. The sugar consumed was determined by Shaffer and Somogi's method.[4] The yield of the fumaric acid was calculated on the basis of sugar consumed.

(*k*) *Confirmatory test of fumaric acid*

Confirmation of fumaric acid produced was done by M.P., mixed M.P. and decolorization of acidified potassium permaganate solution in the cold.

## RESULTS AND DISCUSSION

The yield of fumaric acid produced by *R nigricans* from glucose and by *R arrhizus* from different carbohydrates/carbohydrate sources are given in Table 1. It will be seen from the Table that higher yield is obtained from glucose as compared to cane sugar and other carbohydrate sources using *R arrhizus*. It will be further seen from this Table that very poor yield (7 per cent) of fumaric acid is obtained from 50 : 50 deionised bagasse hydrolysate and glucose medium under similar conditions of the experiment. No production of fumaric acid took place when only deionised bagasse hydrolysate medium supplemented with various nutrients was used. Some experiments were also done using

TABLE 1

EFFECT OF DIFFERENT CARBOHYDRATE SOURCES ON THE YIELD OF FUMARIC ACID BY STATIONARY SURFACE CULTURE METHOD

| Organism | Substrate | Incubation period (in days) | Initial sugar concentration (%) | Sugar consumed (%) | Fumaric acid produced (gm/100ml) | Yield of fumaric acid (%) |
|---|---|---|---|---|---|---|
| Rhizopus nigricans | glucose | 15 | 10 | 85·5 | 2·2 | 25·73 |
| *Rhizopus arrhizus* | glucose | 15 | 12 | 87 | 3·9 | 33·5 |
| | Cane sugar | 15 | 12 | 87·5 | 2·20 | 21·0 |
| | Cane sugar | 18 | 12 | 100 | 3·30 | 27·5 |
| | Clarified molasses | 18 | 12 | 99·00 | nil | nil |
| | 50 : deionized bagasse hydrolysate +glucose | 18 | 12 | 94·5 | 0·81 | 7·2 |
| | Deionized bagasse hydrolysate | 18 | 12 | 85·0 | nil | nil |
| | Xylose | 18 | 12 | 86.0 | nil | nil |

12 per cent xylose as a carbohydrate source and fumaric acid produced was practically nil. However, the growth of the fungus was apparently normal as with glucose medium. This indicates that *R arrhizus* is not able to utilize pentose sugars either as such or those present in bagasse hydrolysate.

Experiments were also carried out separately with molasses, clarified by different methods[5] using *R arrhizus* but the formation of fumaric acid was not detected.

Table 2 summarises the results of the study on the effect of different concentrations of sugars on the yield of fumaric acid. It will be seen from the data that with 12 per cent glucose, *R arrhizus* has given the highest yield *i.e.* 33 per cent fumaric acid and the glucose is better utilized by this organism than cane sugar Foster[6] reported that *R nigricans* does not have *invertase enzyme.* This may be the reason for non-utilization of cane sugar by this organism.

The effect of different concentrations of $KH_2PO_4$ on the yield of fumaric acid by stationary surface culture method obtained from *R. arrhizus* is shown in Table 3. It has been noted that maximum production of fumaric acid takes place when concentration of $KH_2PO_4$ is 0·03 per cent.

The effect of incubation period on the yield of fumaric acid from glucose and cane sugar are given in Table 4. The data indicated that maximum yield of fumaric acid is obtained when the incubation period is 15 days in the case of glucose and 18 days in the case of cane sugar.

TABLE 2

THE EFFECT OF DIFFERENT CONCENTRATIONS OF CARBOHYDRATES ON THE YIELD OF FUMARIC ACID (INCUBATION PERIOD 15 DAYS)

| Organism | Substrate | Initial sugar concentration (%) | Sugar consumed (%) | Fumaric acid produced (gm/100ml) | Yield of fumaric acid (%) |
|---|---|---|---|---|---|
| *Rhizopus nigricans* | glucose | 4 | 95 | nil | nil |
| | | 6 | 96 | nil | |
| | | 8 | 86 | nil | |
| | | 10 | 96·3 | 2·5 | 25·80 |
| | glucose | 6 | 95·5 | 0·7 | 12·7 |
| | | 8 | 95·5 | 1·12 | 14·0 |
| | | 10 | 94·8 | 2·52 | 26·9 |
| | | 12 | 86·50 | 3·86 | 33·3 |
| | | 15 | 95·20 | 4·12 | 28·8 |
| *Rhizopus arrhizus* | cane sugar | 6 | 96 | 0·88 | 14·60 |
| | | 8 | 98·5 | 1·20 | 15·20 |
| | | 10 | 96·2 | 2·32 | 24·10 |
| | | 12 | 100 | 3·3 | 27·50 |
| | | 15 | 92·6 | 2·8 | 20·10 |

## SUMMARY

*R nigricans* and *R arrhizus* were used for the production of fumaric acid from different carbohydrate sources. Twenty-six and 33 per cent yield of fumaric acid were obtained with 10 and 12 per cent glucose medium respectively in 15 days at 33°C by stationary surface culture method.

Of the two fungi *R arrhizus* gave higher yield and hence this organism was selected for detailed studies. This fungus was acclimatized on media containing (*i*) 50 : 50 glucose and deionised bagasse hydrolysate, (*ii*) deionised bagasse hydrolysate, (*iii*) clarified cane molasses. 7 per cent yield of fumaric acid was obtained from 50 : 50 glucose and deionised bagasse but no production of fumaric acid took place when only deionised bagasse hydrolysate medium supplemented with various nutrients was used. Experiments were also done using 12 per cent xylose as a substrate. In such cases the growth of the fungus was apparently normal as with glucose, however, the yield of fumaric acid was practically nil. The above findings indicate that *R arrhizus* is not able to utilize pentose sugars either as such or those present in bagasse hydrolysate. Experiments were also done separately with classified molasses[5] using *R arrhizus* but fumaric acid was not detected.

TABLE 3

EFFECT OF DIFFERENT CONCENTRATION OF $KH_2PO_4$ ON THE YIELD OF FUMARIC ACID BY STATIONARY SURFACE CULTURE METHOD

| Concentration of $KH_2PO_4$ (%) | Initial glucose concentration (%) | Glucose consumed (%) | Fumaric acid produced (gm/100ml) | Yield of fumaric acid (%) |
|---|---|---|---|---|
| 0·01 | 12 | 89·50 | 1·18 | 10·99 |
| 0·02 | ,, | 95·5 | 2·47 | 21·20 |
| 0·03 | ,, | 95 | 3·64 | 32·60 |
| 0·04 | ,, | 95·25 | 1·57 | 13·00 |
| 0·05 | ,, | 95·75 | 1·87 | 16·00 |

Fungus *R. arrhizus*
Incubation period 15 days
Temp. 33°C

TABLE 4

EFFECT OF INCUBATION PERIOD ON THE YIELD OF FUMARIC ACID

| Incubation period (days) | Substrate | Initial sugar concentration (%) | Sugar consumed (%) | Fumaric acid produced (gm/100ml) | Yield of fumaric acid % |
|---|---|---|---|---|---|
| 15 | glucose | 12 | 86·5 | 3·5 | 32·30 |
| | | | 95·75 | 3·80 | 33·00 |
| 18 | | 12 | 100 | 3·80 | 3·16 |
| | | | 98·75 | 3·50 | 29·6 |
| 15 | cane sugar | 12 | 85·25 | 2·10 | 20·50 |
| | | | 87·5 | 2·20 | 20·95 |
| 18 | | 12 | 100 | 3·00 | 25·0 |
| | | | 100 | 3·1 | 25·80 |

Fungus *R. arrhizus*
Temp. 33°C.

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