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Research Article

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Effect of Different Factors on Organic Acid Production by *Sclerotium rolfsii*

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ABSTRACT

In the present research work effect of different culture media, temperatures and pH on organic acid (oxalic acid) production by Sclerotium rolfsii was studied. Qualitative estimation of organic acid was assayed by Foster and Davis method with some modification.

To find out the best suitable medium for production of organic acid, total ten media, viz., Asthana and Hawker's, Basal mucor, Brown's, Czapek's, Dextrose-asparagine phosphate, Elliot's, Fernando's, Glucose-dox, Glucose-nitrate and Potato-dextrose, agar media were tried. Out of above ten media, only six media i.e., Elliot's, Fernando's, Basal mucor, Brown's, Dextrose-asparagine phosphate and Potato-dextrose agar media were proved to be producer of organic acid. Amongst, above said six media, the Potato-dextrose medium was found to be the best, as maximum accumulation of organic acid was recorded in this medium followed by Dextrose-asparagine phosphate medium. The Potato-dextrose medium was selected for further study for organic acid production by Sclerotium rolfsii.

To study the effect of various temperatures, viz., 15, 20, 25, 30, 35, 40 and 45ºC, on the production of organic acid, the fungus was cultured on Potato-dextrose agar medium and incubated at above said different temperatures. When Sclerotium rolfsii was incubated at very low temperature i.e., 15ºC, a little amount of organic acid was detected. The accumulation of organic acid was increased gradually by with increase in temperature up to 30ºC. Above 30ºC, further increase in temperature have no effect on the production of organic acid. Therefore, 30ºC temperature was found to be the best and optimum for the production of organic acid.

The effect of different pH values i.e., pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 on the production of organic acid was studied and results revealed that the isolated pathogen Sclerotium rolfsii was able to produce organic acid at very wide range of pH i.e., pH 3.0 to pH 9.0 (i.e., extreme acedic to alkaline pH). The pH 5.0 was proved to be the best for the production of organic acid, as maximum accumulation was detected when Sclerotium rolfsii was cultured on the medium having pH 5.0.

Keywords : *Sclerotium rolfsii, Organic acid, Agar media, Temperature, pH.*

INTRODUCTION

Since earliest time, fungi have been known primarily as destructive agent due to their deleterious action on food stuff. It was Louis pasteure (1879) who said "We are convinced a day will come when moulds (fungi) will be utilized in certain industrial operations on account of their power of destroying organic matter." Then he immediately proceeded to what he had in mind and reported the acetification of alcohol in vinegar process and the production of gallic acid (organic acid) by action of fungi on wet-gall nuts.

Fungi thus serves a dual purpose firstly they proves to good agent for disposed of waste materials by their saprophytic ability, secondly due to their metabolic activity they produced a number of useful products. Fungi as a group, contain widely diverse type of microorganisms ranging from microscopic to easily visible mushrooms.

Their metabolic activity are similarly diverse and literally hundreds of products are produced during their physiological process. Most of fungi possess a wealth of metabolic equipment that produced various type of useful commercial products of daily use such as enzymes, antibiotics, protein, fats, amino acids, organic acids etc.

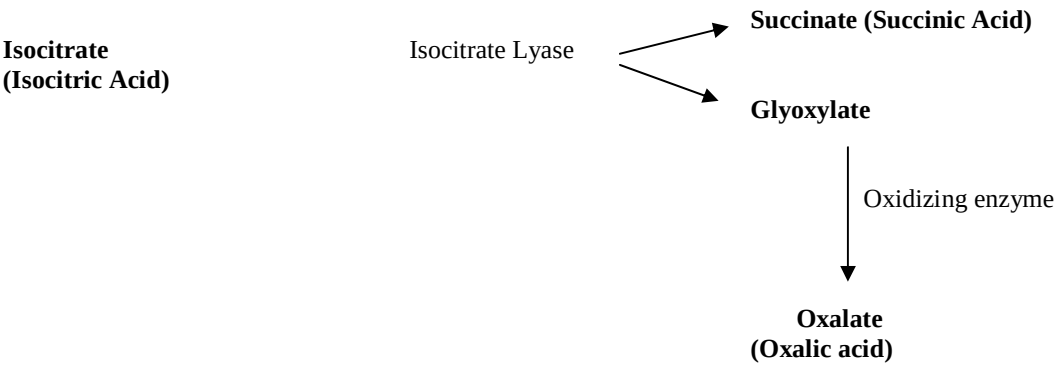
Organic acid one of the important metabolite produced by fungi as result of dissimilation of carbohydrates[12,20] . The important organic acids produced by fungi are citric acid, fumaric acid, malic acid, succinic acid, itaconic acid, oxalic acid etc. the various type of organic acids, forms a vital part of our civilization and they are widely used for purpose of medicines, food technology, beverage industries, plastics industries dyeing and calico printing, silvering agent and also used as ingredient of engraving inks etc[9,11]. The production of organic acid particularly citric and oxalic acid, on a commercial basis has been an important achievement in the field of industrial microbiology. Amongst organic acid produced by microorganisms, oxalic acid, is one of the important. It is an dicorboxylic acid of aliphatic series.

Long back, Thom and Currie[24], demonstrated the production of oxalic acid by strain of *Aspergillus niger*. The importance of oxalic acid production during pathogensis of *Sclerotium rolfsii* has been stressed by Higgins[8] ; Kritzman *et.al*.[10]; Punja[18]; Dutton and Evans[6]; Paramasivan *et.al*.[17].

After that several investigators have reported the production of oxalic acid both in vitro and vivo. The co-relation of oxalic acid production with pathogenicity in *Sclerotium rolfsii* has been studied in detaild by Bateman and Beer[2], Maxwell and Bateman[13,14], Mehrotra and Claudieus[16] & many others. They demonstrated that the presence of oxalic acid coupled with disease producing enzymes (i.e., cellulase & pectolytic enzymes), always resulted in more severity of disease, thus oxalic acid play an important role in production of disease symptoms.

The biosynthesis of oxalic acid by *Sclerotium rolfsii* through glyoxylate by pass of tricarboxylic acid cycle has been demonstrated by Maxwell and Bateman[14]. They have shown that *Sclerotium rolfsii*, able to produced necessary enzymes which helps in accumulation of oxalic acid. According to them, the biosynthesis of oxalic acid in *Sclerotium rolfsii* involves the cleavage of isocitric acid (formed in TCA cycle during respiration) in to succinate (Succinic acid) and glyoxylate (glyoxylic acid) in the presence of enzyme isocitrate lyase. Later on in presence of oxidizing enzyme, glyoxylate converted into oxalic acid.

Schematic representation is[14] as follows :



The estimation of oxalic acid was determine in various isolates of *Sclerotium rolfsii* in vitro condition by Ansari and Agnihotri[1]. Recently the variation in oxalic acid production by groundnut isolates of *Sclerotium rolfsii* was determine by Saraswathi and Jaya Madhuri[21].

Keeping the above facts in mind, the present work was undertaken to study the production of organic acid (as oxalic acid) by *Sclerotium rolfsii* in vitro condition.

MATERIALS AND METHODS

Isolation of Pathogen and Maintenance of Culture :

Brinjal plants showing foot-rot disease symptoms were collected and used for isolation of *Sclerotium rolfsii* Sacc. pure culture was maintained on potato-dextrose agar agar salnts under refrigeration at 4ºC. The slants were freshly made once a month.

Preparation of stock solution of indicator :

500 mg. of bromcresol green was taken as an indicator and dissolved in 10 ml of 0.1 N NaOH solution. After dissolving, the indicator solution was diluted to 500 ml by adding more distilled water. This solution was always used as an indicator.

The detection and assaying of organic acid :

For the detection and qualitative assaying of organic acid, the method proposed by Foster and Davis[7] was employed with some modification. Twenty ml. of melted medium was poured in to each petridish and inoculated by placing agar disc inoculums in the centre. Inoculated petridishes incubated for 24, 48 and 72 hours, in incubator at 30ºC (except temperature experiment). After incubation, these petridishes were directly tested for organic acid detection. For the detection of organic acid, bromcresol green indicator solution was spreaded over the surface of agar. On applying the indicator over agar surface, a bright yellow zone appeared around the colony against deep blue background. The diameter of the yellow zone was measured in mm and the qualitative estimation of organic acid assay by substrating the diameter of inoculum from the diameter of yellow zone was done.

Different factors and organic acid production :

a. Different culture media :

To see the effect of different agar media on organic acid production the following ten media of composition (g/l) were tested.

1. **Asthana and Hawker's :**
   Glucose 5, KNO_3 3.5, KH_2PO_4 1.75, $MgSO_4$. $7H_2O$ 0.75, Agar-agar 20.0, Distilled Water to 1 L.

2. **Basal mucor ;**
   Dextrose 10, Agar-agar 20.0 Asparagine 2, KH_2PO_4 0.5, $MgSO_4.7H_2O$ 0.25, Thiamine chloride 0.5, Agar-agar 20.0, Distilled Water to 1 L.

3. **Brown's :**
   $MgSO_4.7H_2O$ 0.75, KH_2PO_4 1.25, Asparagine 2, Dextrose 20, Starch 10,

   Agar-agar 20.0, Distilled Water to 1 L.

4. **Czapek's :**
   $NaNO_3$ 2, KH_2PO_4 1, $MgSO_4.7H_2O$ 0.5, KCl 0.5, $FeSO_4.7H_2O$ 0.01, Sucrose

   30, Agar-agar 20.0, Distilled Water to 1 L.

5. **Dextrose-asparagine phosphate :**
   Dextrose 30, $MgSO_4.7H_2O$ 0.5, Asparagine 1, KH_2PO_4 1.5, Agar-agar 20.0, Distilled Water to 1 L.

6. **Elliot's :**
   Dextrose 5, Asparagine 1, Sodium Carbonate 1.06, $MgSO_4.7H_2O$ 0.5,

   KH_2PO_4 1.36, Agar-agar 20.0, Distilled Water to 1 L.

7. **Fernando's :**
   $MgSO_4$ 5, KH_2PO_4 6.8, Asparagine 5, Glucose 15, Agar-agar 20.0, Distilled Water to 1 L.

8. **Glucose-dox :**
   $MgSO_4.7H_2O$ 0.5, KH_2PO_4 1, $FeSO_4.7H_2O$ 0.01, $NaNO_3$ 2, KCl 0.5,

   Glucose 15, Agar-agar 20.0, Distilled Water to 1 L.

9. **Glucose-nitrate :**
   Glucose 10, $NaNO_3$ 1, KH_2PO_4 1, Agar-agar 20.0, Distilled Water to 1 L.

10. Potato dextrose :

Peeled potato slices 200, Dextrose 20, Agar-agar 20.0, Distilled Water to 1L.

For the production of organic acid 20 ml. of each above medium was poured in petridishes after proper sterilization. Each set was run in triplicate. After inoculation, these petridishes were kept for 24, 48 and 72 hours in incubator at 30ºC. After incubation, bromcresol green solution indicator was applied around the colony with the help of cotton, organic acid producers, gave a clear bright yellow zone around the colony which was measured.

b. Different temperatures :

To study the effect of different temperatures viz., 15, 20, 25, 30, 35, 40 and 45ºC on production of organic acid, by *Sclerolium rolfsii*, 20 ml of Potato-dextrose agar medium was used and the pathogen was inoculated in petridish. A set of three petridishes, were kept in different incubators adjusted at different temperature i.e., 15ºC, 20ºC, 25ºC, 30ºC, 35ºC, 40ºC and 45ºC. After 24, 48 and 72 hours of incubation, each set of inoculated petridishes were assayed for determination of organic acid production.

c. Different pH :

The Potato-dextrose agar medium was adjusted at different pH values i.e., pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 with the help of 0.1 N HCl or 0.1 NaOH solutions. After inoculation with *Sclerotium rolfsii*, petridishes were incubated at 30ºC for 24, 48 and 72 hours. Each set was run in triplicate. At the end of each incubation period, petridishes were assayed for determination of organic acid production.

RESULTS AND DISCUSSION

Effect of different culture media on the production of organic acid :

The effect of ten different media viz., Asthana and Hawker's, Basal mucor, Brown's Czapek's, Dextrose-asparagine phosphate, Elliot's, Fernando's, Glucose-dox, Glucose-nitrate and Potato-dextrose agar media were tried to investigate the production of organic acid (oxalic acid). The organic acid (oxalic acid) was qualitatively estimated in all the above ten taken media and results are presented in Table 1 and Plate 1. In organic acid producer medium, after testing, a clear bright yellow zone was observed around the colony (Plate 1 and 2).

Table 1 : Effect of different agar media on the production of organic acid by *Sclerotium rolfsii*.

Media	Organic acid production (Width of yellow zone in mm around growing colony)*		
	Hours after inoculation		
	24	48	72
Asthana and Hawker's	0.0	0.0	0.0
Basal mucor	12.0	30.3	45.2
Brown's	14.2	33.4	48.1
Czapek's	0.0	0.0	0.0
Dextrose-asparagine phosphate	20.5	37.2	57.5
Elliot's	15.3	21.1	23.6
Fernando's	20.4	33.6	42.4
Glucose-dox	0.0	0.0	0.0
Glucose-nitrate	0.0	0.0	0.0
Potato-dextrose	30.2	66.5	82.0

*** After deducting the inoculum disc of 8.0 mm diameter.**

From the results, it is evident that out of ten agar media, only six media namely Basal mucor, Brown's, Dextrose-asparagine phosphate, Elliot's Fernando's and Potato-dextrose were found to be as producer of organic acid and remaining four agar media i.e., Asthana and Hawker's, Czapek's, Glucose-dox and Glucose nitrate were recorded as non-producer of organic acid.

Chaurasia[5] has reported that Asthana and Hawker's, Czapek's, Glucose-dox and Glucose-nitrate agar media did not support the growth as in all these four media *sclerotium rolfsii* was unable to grow. Perhaps nitrogen source and other ingradients of the medium may interfered with the growth of pathogen and production of organic acid. Several workers like Ritter[19], Thornton[25], Sarbhoy[22,23] and Bilgrami[3] having also similar opinion as they have reported that nitrate containing media are very toxic to several members of microorganisms. Amongst, six organic acid producer media, Potato-dextrose agar medium found to be the best as maximum accumulation of organic acid was observed and Dextrose-asparagine phosphate medium was ranked next as it also gave a appreciable yield of organic acid. the satisfactory production of organic acid was estimated in Basal mucor, Brown's and Fernando's agar media while Elliot's agar medium was found to poor in this respect. The relative degree of production of organic acid amongs the organic acid producer media are as follows :

Potato-dextrose > Dextrose-asparagine phosphate > Brown's > Basal mucor > Fernando's > Elliot's agar medium.

Plate 1. showing the effect of following agar media on the production of organic acid (After 72 hours of inoculation).

1. Asthana and Hawker's medium.

2. Basal mucor medium.

3. Brown's medium.

4. Czapeks medium.

5. Dextrose-asparagine phosphate medium.

6. Elliot's medium.

7. Fernando's medium.

8. Glucose-dox medium.

9. Glucose-nitrate medium.

10. Potato-dextrose medium.

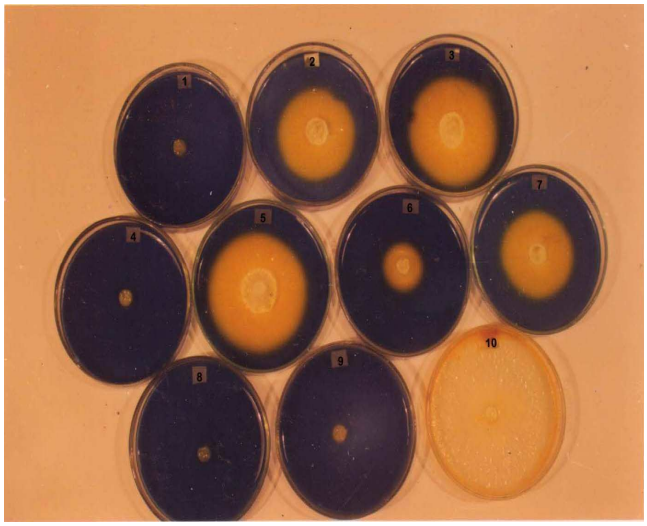
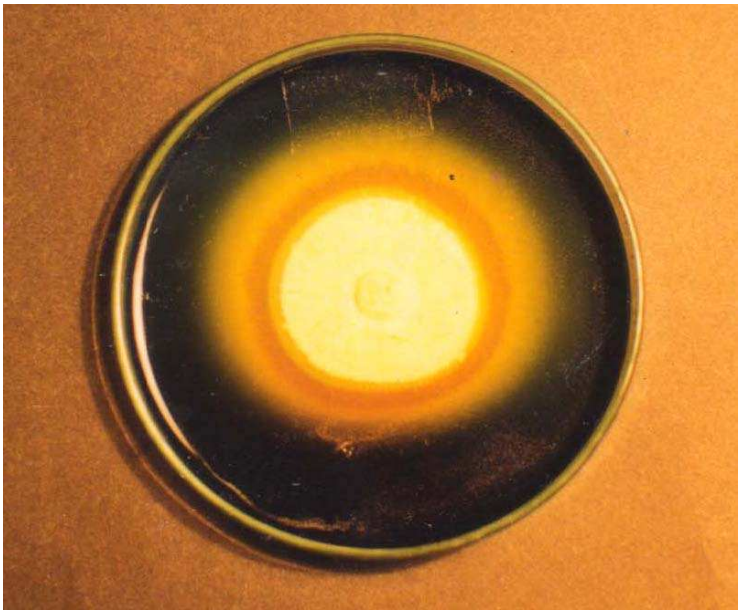


Plate 2. showing organic acid production on Potato–dextrose agar medium (after 48 hours of inoculation).



In the light of above results it can be concluded that Potato-dextrose agar medium was found to be the best, as maximum amount of organic acid was estimated when *Sclerotium rolfsii* was cultured on Potato-dextrose agar medium. Therefore, Potato-dextrose agar medium was selected for the further investigations.

Effect of different temperatures on the production of organic acid :

The effect of different temperatures viz., 15, 20, 25, 30, 35, 40 and 45ºC were tried to study their influence on the production of organic acid (oxalic acid) by *Sclerotium rolfsii*. The results obtained in present investigation are tabulated in Table 2.

Table 2 : Effect of different temperatures on the production of organic acid by *Sclerotium rolfsii*.

Temperature (ºC)	Organic acid production (Width of yellow zone in mm around growing colony)*		
	Hours after inoculation		
	24	48	72
15	0.0	5.0	11.5
20	0.0	16.5	40.3
25	18.5	42.4	68.4
30	30.2	66.5	82.0
35	19.5	48.0	77.5
40	0.0	0.0	0.0
45	0.0	0.0	0.0

*** After deducting the inoculum disc of 8.0 mm diameter.**

When the *Sclerotium rolfsii* was incubated at lowest temperature, i.e., at 15ºC temperature, it was able to produce organic acid in a very little amount. The production of organic acid was regularly increased with increase in temperature upto 30ºC. Further increase in temperature i.e., at 35ºC, having no effect on the production of organic acid but it resulted in slight decline. Higher range of temperature i.e., 40 and 45ºC, have been found to be detrimental for *Sclerotium rolfsii*, hence no trace of organic acid was detected around the inoculum. Chaurasia *et.al*.[5] has reported that higher temperature i.e., 40 and 45°C were found to be unfavorable for the growth of *Sclerotium rolfsii* and in these temperature the pathogen was unable to grow. These findings clearly indicate the important role of temperature in the production of orgnic acid.

Results of present investigation were confirm by Mehrotra and Tondon[15], as they have resulted the 30ºC temperature found to be optimum and acid formation was found only within the range of 25ºC to 35ºC.

On the whole it can be concluded that with increase in temperature, the production of organic acid increased up to 30ºC and further increase in temperature have no effect on the production of organic acid. therefore, 30ºC temperature found to be optimum and suitable for the production of organic acid.

Effect of different pH on the production of organic acid :

To investigate the effect of pH, the Potato-dextrose agar medium was adjusted at different pH values i.e., from pH 3.0 to 9.0 over a interval of pH 1.0. The production of organic acid was qualitatively estimated by growing the colony of *Sclerotium rolfsii*, on Potato-dextrose agar medium of different pH value and results are presented in Table 3.

Table 3: Effect of different pH on the production of organic acid by *Sclerotium rolfsii*.

pH	Organic acid production (Width of yellow zone in mm around growing colony)*		
	Hours after inoculation		
	24	48	72
3.0	3.5	17.2	38.0
4.0	19.5	41.5	67.5
5.0	30.2	66.5	82.0
6.0	24.6	52.2	78.0
7.0	18.5	41.0	61.5
8.0	17.5	36.5	56.3
9.0	14.3	30.4	45.6

*** After deducting the inoculum disc of 8.0 mm diameter**

From the data recorded in Table 3, it was observed that a considerable sufficient amount of organic acid was produced and detected when *Sclerotium rolfsii* grew on that media which having a slight acedic pH i.e., 4.0 to 6.0 (acidic pH range) in comparision to alkaline pH range (above pH 7.0). At extreme acidic pH value i.e., pH 3.0, production of organic acid was found to less and further increase in pH value from pH 3.0, the production was gradually increased with increase in pH upto 5.0 which was recorded as optimum pH for production of organic acid. Above pH 5.0, further increase in pH, have no effect but rather resulted in gradual decrease in the production of organic acid. The effect of pH on the organic acid production indicate that the organic acid production is active in the pH range 3.0 to 9.0. This suggest that the organic acid production would be useful in process that require by range of pH change from highly acedic to slightly alkaline range and vice-versa. Similar results have also been recorded by Chaurasia[4] in case of amylase production of *Sclerotium rolfsii*.

On the whole it can be concluded that the maximum production of organic acid was estimated in that medium, which was adjusted at pH 5.0, therefore, pH 5.0 was found to be the best and most favourable for the production of organic acid.

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