Int J Pharm Bio Sci 2013 Jan; 4(1): (B) 828 - 834

Research Article **Bio chemistry**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

OXALIC ACID PRODUCTION BY *ASPERGILLUS NIGER*

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ABSTRACT

Oxalic acid produced by *Aspergillus niger* under submerged conditions in this work. Optimization done with different sources of carbon, nitrogen, minerals and at different pH then biomass production done of *Aspergillus niger* under submerged conditions for 7 days at room temperature. Further supernatant taken for titration and mycelium for oxaloacetate acetylhydrolase determination by SDS-PAGE.

KEY WORD: **Oxalic acid, *Aspergillus niger,*Submerged fermentation, SDS-PAGE**

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INTRODUCTION

Oxalic acid is produced by a variety of fungi, including saprophytic and phytopathogenic species[2]. In phytopathogenic fungi it is believed to play a role in pathogenesis, facilitating plant cell wall degradation. In saprophytic Species, such as *Aspergillus niger*, the role of oxalic acid production is less clear, but may also be related to mobilizing substrates from plant cell wall polysaccharides, e.g. pectin[14]. In *Aspergillus niger*[4,11,10] and in a number of other fungi[2], as well as in some *Streptomyces* species[5], the current evidence favours production of oxalic acid by a Mn^{2+} dependent enzyme, oxaloacetate acetylhydrolase (EC 3.7.1.1). In *Aspergillus niger* this enzyme is localized in the cytoplasm[6], where it catalyses the following reaction: Oxaloacetate → oxalate + acetate. Second pathway[1] should exist in *Aspergillus niger* which generates oxalate from pentoses via glycolate and glyoxylate as intermediates. This route requires operation of glyoxylate dehydrogenase as the final step to oxidize glyoxylate to oxalate, but attempts to measure glyoxylate oxidizing enzymes in extracts of *Aspergillus niger* that produced oxalate were unsuccessful[11]. The biosynthesis of oxalic acid seems to fit the general strategy of *Aspergillus niger* and other fungi to acidify their environment via an extracellular process involving glucose oxidase, by secreting organic acids which first accumulate intracellularly or by a combination of these processes. *Aspergillus niger* is a very efficient oxalic acid producer. Production of 13 g oxalic acid/l from 20 g sugar/l in 45h [15]. Production of 38 g oxalic acid. Which is close to the solubility of sodium oxalate[13] using a fed-batch process at pH 6 with sucrose as the carbon source. Oxalic acid produced by this pathogenic fungus played an essential role in its pathogenic capabilities[12], another pathogenic fungus infecting a wide range of plant species is *Sclerotinia sclerotiorum*. During infection, the fungus produces high levels of a necrosis phytotoxin identified as oxalic acid[7]. The role of oxalic acid in the pathogenicity process is still unclear. However, oxalic acid may have a number of functions in the infection process including chelating calcium from the cell was thus making the pectic fraction more available to fungal hydrolases, and providing an acid pH needed for maximum activity of the wall degrading enzymes released by the pathogenic fungus[8].

MATERIALS & METHODS

Isolation and Identification

Make 100 ml potato dextrose agar media, and pour it in four Petri dish. After solidify spread bread in the Petri dish, and after three days able to see over their culture of *Aspergillus niger* and identified it by Lactophenol Cotton Blue Staining.

Production media

The production of oxalic acid from *Aspergillus niger* was carried out in medium which have composition Glucose 105.5g, Sodium nitrate 1.5g, Dipotassium dihydrogen orthophosphate 0.5g, Magnesium chloride 0.025g, Potassium chloride 0.025g, Yeast extract 1.6g, Distilled water 1000ml, pH 6 ± 0.5.

Optimization of production media

Rice bran, Potato, Sweet potato, Tomato juice and Fruit pulp as a carbon sources; Peptone, Yeast extract, Beef extract, Ammonium nitrate, Ammonium sulphate and Potassium nitrate as a nitrogen sources; Different concentration of dipotassium dihydrogen orthophosphate 0.05, 0.10, 0.15, 0.20 and 0.30 mM as a mineral sources and Different pH 1, 3, 5, 7, 9 and 11 were taken made 100 ml production media for each. Mycelium yield was determined after 7 days of incubation at room temperature.

Preparation of cell extracts and enzyme assay[10]

Mycelium was collected from a culture sample by filtration under vacuum, washed three times with approximately 50 ml, 10 mM potassium phosphate buffer pH 7 and frozen in liquid nitrogen. For each sample

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approximately 0.5 g of the frozen mycelium was powdered using a micro-dismembrator and suspended in 1 ml extraction buffer at 0°C. Oxaloacetate acetylhydrolase was extracted in 50 mM potassium phosphate pH 7 containing 0.5 mM EDTA, 5 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 10% (v/v) glycerol. Following centrifugation at 15,000 rpm for 5 min enzyme activities were assayed in the resulting supernatant. Oxaloacetate acetylhydrolase activity was measured using direct optical determination of oxaloacetate at 255 nm[10].

Purification and determination of oxaloacetate acetylhydrolase[3, 9]

Disruption of mycelium was done as described under Preparation of cell extracts and enzyme assays. The resulting suspension was centrifuged at 10000 rpm for 10 min at 4°C. To the supernatant, NH_2SO_4 was added to 40% saturation. Precipitation of protein was allowed to occur for 20 min at 4°C with gentle mixing. To the supernatant obtained after centrifugation for 10 min at 10000 g and 4°C, NH_2SO_4 was added to obtain 50% saturation. Following 20 min incubation at 4°C and another centrifugation step the precipitated protein, which contained oxaloacetate acetylhydrolase[3]. Denaturing electrophoresis in 10% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS was performed[9].

Estimation of oxalic acid by titration

Dilute a sample 5ml in a 45ml volumetric flask using water and pipette 5ml of that diluted sample into a boiling flask and add 50% sulfuric acid heat it 65-70°C then titrate with N/10 KMnO4. Volume of KMnO4 (N/10) x 6.3 = g/l of oxalic acid. Where 6.3 is factor.

RESULTS AND DISCUSSION

Identification

Lactophenol cotton blue staining: *Aspergillus niger*, Delicate blue hyphae and fruiting structures with a pale blue background (Figure 1 & 2).

Optimization

Aspergillus niger culture was optimized with different sources of carbon, nitrogen, mineral concentration and at different pH. The results of optimization were, maximum oxalic acid observed when sweet potato used as a carbon source (Table no. 1), peptone used as a nitrogen source (Table no. 2), Dipotassium dihydrogen orthphophate with 0.10mM as a mineral source (Table no. 3) and at pH 7 (Table no. 4).

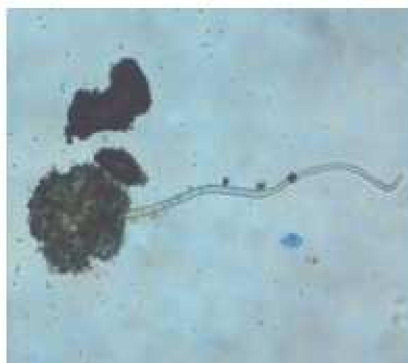

Figure 1 & 2

Asperillus niger on potato dextrose agar media and microscopic view

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Table no. 1
Optimization of carbon sources

Carbon Sources	Mycelium dry weight (g/100ml)	Oxalic acid (g/l)
Potato	3.47	**2.52**
Sweet potato	6.27	**3.78**
Rice bran	0.47	**1.89**
Fruit pulp	6.42	**3.15**
Sugarcane baggase	0.09	**1.26**
Tomato juice	5.05	**2.52**

Table no. 2
Optimization of nitrogen sources

Nitrogen Sources	Mycelium dry weight (g/100ml)	Oxalic acid (g/l)
Beef extract	9.75	**4.41**
Yeast extract	11.35	**2.52**
Ammonium nitrate	12.79	**3.15**
Ammonium sulphate	2.03	**3.78**
Peptone	15.04	**5.04**
Potassium nitrate	11.17	**4.41**

Table no. 3
Optimization of mineral sources

K2H2PO4 (mM)	Mycelium dry weight (g/100ml)	Oxalic acid (g/l)
0.05	11.31	**3.15**
0.10	4.34	**4.41**
0.15	10.92	**1.26**
0.20	12.38	**1.89**
0.25	11.62	**1.89**
0.30	10.22	**1.89**

Table no. 4
Optimization of pH

pH	Mycelium dry weight (g/100ml)	Oxalic acid (g/l)
1	12.93	**1.26**
3	15.82	**1.89**
5	12.84	**1.26**
7	17.56	**3.15**
9	12.07	**1.15**
11	8.30	**2.52**

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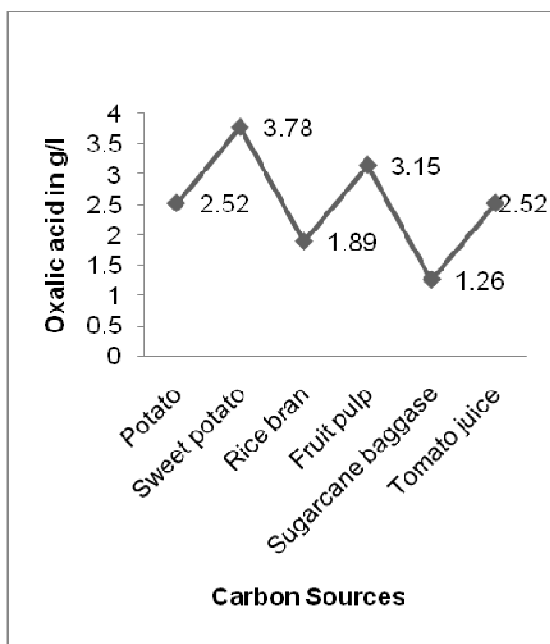
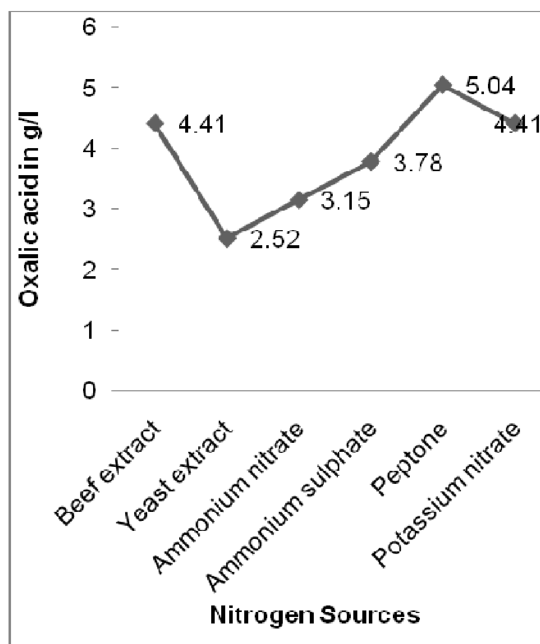
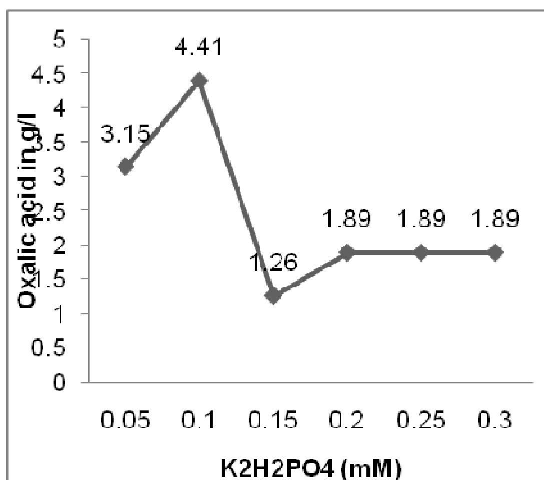


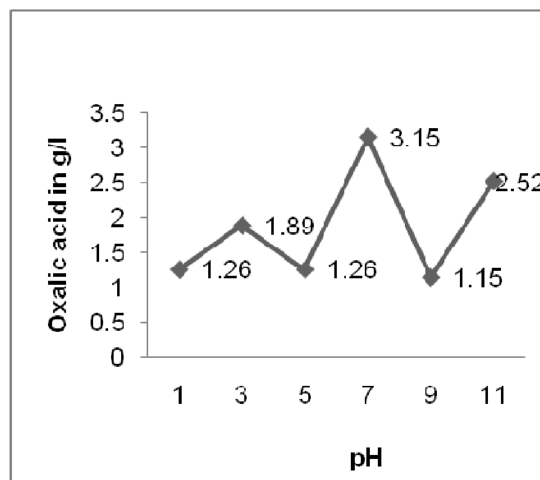
Graph 1



Graph 2



Graph 3



Graph 4

Graphs (1, 2, 3 & 4) showing Optimization of Carbon, Nitrogen, Mineral sources & pH repectively

SDS-PAGE

Molecular mass markers were used phosphorylase b (92.5 kDa), BSA (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), Trypsin inhibitor (22kDa) and Lysozyme (14kDa) in SDS-PAGE and determined 34 kDa molecular weight of oxaloacetate acetylhyrolase. Most of the data available on oxalic acid production by *Aspergillus niger* are in favour of oxaloacetate acetylhydrolase being the only enzyme responsible for oxalic acid production. 2% glucose as a carbon source, an increase in the $NaNO_3$ concentration from 6 to 60 mM resulted in an increase of molar yield from 0.5 to 0.54, with a concomitant increase in dry weight from 3.2 to 4.2 g/l. Using 28 mM of NH_4Cl instead of $NaNO_3$ the molar yield was 0.5 (dry weight 5.5 g/l)[11]. Thus, the type and concentration of the nitrogen source did not affect the molar yield of oxalic acid very

much and production of 4.3 - 6 .3 oxalic acid g/l from 50 glucose g/l while our data showed maximum production of oxalic acid was with sweet potato (3.78g/l) as a carbon source with 6.27g mycelium dry weight and nitrogen source peptone (5.04g/l) with 15.04g mycelium dry weight after 7 days fermentation at room temperature. The result also shows that maximum production of oxalic acid was at pH 7 (17.56g mycelium dry weight). Concentration of dipotassium dihydrogen orthphophate, production of oxalic acid was the best with 0.10mM conc. (4.41g/l). The enzyme oxaloacetate acetylhydrolase responsible for oxalate formation, this enzyme cleaves oxaloacetate[3], oxalate & acetate in *Aspergillus niger* and protein estimation by SDS-PAGE that means it's capable for splitting the oxaloacatate in this determined also.

CONCLUSION

The main conclusion from the work is that various wastes containing free glucose or sugars are polluting agents and their conversion to useful products, such as organic acids through fermentation, may be considered as an effective measure toward abatement of environmental pollution. The *Aspergillus niger* strains are better because of its ability to converting polluting sugars to oxalic acid.

ACKNOWLEDGEMENT

The work described in this paper was done as M.Sc. Biochemistry dissertation. The author would like to thank to Head of Department of Biochemistry, Bundelkhand University, Jhansi, India for facilities provided.

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