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Optimization and production of uricase enzyme from *Aspergillus niger* isolated from Mangrove sediment

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Aspergillus niger,
mangrove sediment,
gout,
urate oxidase.

Abstract

The present study was on isolation of a potent fungal strain from mangrove sediment for the optimization and production of uricase enzyme. Among the 6 fungal strains isolated, *Aspergillus niger* was found to be a potent uricase producer and it was further used for the optimization studies for uricase production. The optimum cultural parameters that were found to be ideal for the higher uricase production by *A. niger* were incubation time – 96hrs, agitation speed – 150 rpm, pH - 8, temperature – 30^{o}C, salinity – 2%, sucrose – 10% (w/v), uric acid - 0.4% (w/v). The observed maximum biomass and uricase enzyme activity for the ideal parameters were as given in brackets at pH-8 (6.2g/L and 143 U/ml/min), 30^{o}C (5.8 g/L and 140 U/ml/min), 2% NaCl (6.3g/L and 140U/ml/min), sucrose as a carbon source (6.1g/l and 144U/ml/min), 10% sucrose (6g/L and 141 U/ml/min), uric acid as nitrogen source (6.4g/L and 140 U/ml/min) and 0.4% uric acid (5.9g/L and uricase activity of 140 U/ml/min) were observed respectively. In the present study the maximum biomass and uricase production observed in shake flasks with standard substrate i.e. (10% sucrose and 0.4% uric acid) was 7.18g/L and 162U/ml/min. When poultry waste as a cheaper substrate in mass scale production in shake flask, the biomass and uricase activity observed were respectively 6.7g/L and 135U/ml/min. Similarly in fermentor the biomass and enzyme production using standard and cheaper substrates were 9.6g/L and 232U/ml/min. and 8.4g/L and 189U/ml/min. respectively.

Introduction

Urate oxidase or uricase (urate: oxygen oxidoreductase, EC 1.7.3.3), is an enzyme that catalyzes specifically the oxidation of uric acid to allantoin and plays an important part in nitrogen metabolism (Wakamiya *et al.*, 1994). Higher primates (apes and humans) lack functional uricase and excrete uric acid as the end product of purine degradation (Friedman *et al.*, 1985). Uricase is an enzyme in the purine degradation pathway that catalyzes the oxidative breakdown of uric acid to allantoin. Uric acid, the primary end-product of purine metabolism is present in biological fluids, including blood and urine. Various medical conditions increase the amount of uric acid in biological fluids. Such conditions can lead to chronic renal diseases, some organic acidemias and diseases like Lesch–Nyhan syndrome. In some individuals, uric acid precipitate, leading to gout symptoms.

This enzyme is widely present in most of the vertebrates (Schiavon *et al.*, 2000) and first observed in bovine

kidney. Various natural sources such as bacteria (Mansour *et al.*, 1996), fungi (Farley and Santosa, 2002) and eukaryotic cells (Montalbini *et al.*, 1997) were found to be uricase producers. Uricase was originally isolated from mammals and recently from microbial sources such as viz., fungi, yeast and bacteria etc.

Several forms of uricase from microorganisms are currently used as diagnostic reagents to detect uric acid. Uricase is used in medicine and clinical biochemistry as a diagnostic reagent for measurement of uric acid in blood and other biological fluids (Adamek *et al.*,1989). Although several microbial sources of uricase have been proposed for this clinical indication, only one has actually been used commercially under the trade mark of uricozyme and isolated and purified from *Aspergillus flavus*. In this back drop, the present study aimed at searching for potential uricase producing fungal sources isolated from Pichavaram mangrove environment.

Materials and Methods

Description of the study area

The study area, Pichavaram mangrove forest (Lat. 11°20' N; Long. 79° 47' E) is located between the Vellar and Coleroon estuaries (Fig. 1).

Fig. 1: Sampling area (Pitchavaram Mangroves)

Collection of mangrove sediment

Sediment samples were collected from Pichavaram mangroves, Tamilnadu, South India by employing an alcohol-sterilized small Peterson grab at about 2m depth. Samples were transferred to the laboratory in an icebox maintained at 4ºC and were processed immediately for the strain isolation.

Isolation of fungi and screening for uricase production

1gm of sediment sample was mixed with 100ml of sterilized 50% aged sea water. 0.1ml each dilutions were plated on Czapek-Dox agar medium plates using spread plate technique. Plates were incubated at 28˚C for 3-7 days. The isolated fungal strains were inoculated into 10ml Czapek-Dox broth medium and were incubated for 28˚C for 3-5 days. After incubation, the mycelia were harvested by filtration. The cell free broth was used to test the extracellular uricase production by using well diffusion assay method on Czapek-Dox agar plate supplemented with 0.3% uric acid. Formation of clear zone around the well was considered as positive for uricase production. Isolate showed maximum zone of clearance was selected as the potent uricase producer and it was used for further study.

Identification of fungus by using lactophenol cotton blue staining

Lactophenol cotton blue stains the fungal cytoplasm and provides a light blue background against which the walls of hyphae can readily be seen. The identification was done by referring the standard books and manual (Ainsworth *et al.*, 1973).

Optimization of cultural conditions of *Aspergillus niger* for biomass and uricase production

Incubation period

Aspergillus niger spores (1 ml of 1.0x 10^6 spores/ml) were inoculated in the Czapek-Dox medium and incubated for different time interval to achieve higher biomass and high rate of uricase production.

The same inoculum concentration was used for the other parameters tested such as agitation, pH, temperature etc., The incubation period was kept as 168 hrs with an interval of 24hrs. As highest biomass and uricase production was observed at 96hrs of incubation, further optimization for each parameter were kept at 96hrs. For each parameter tested biomass and enzyme activity were tested.

Agitation

The different agitation speeds kept were 0 rpm (static), 50 rpm, 100 rpm, 150rpm and 200rpm. All the flasks were incubated for 96hrs. Optimum agitation was maintained for the rest of the parameters tested.

pH

Optimum pH was studied by varying the medium pH such as 4, 5, 6, 7, 8, 9, 10, 11 and 12. The following buffers were used: 100 mM citrate for pH 4.0–6.0, 100 mM phosphate for pH 6.0–8.5 and 100mM borate for pH 8.5–12.

Temperature

Optimum temperature was studied by varying the incubation temperatures at 20°C, 25°C, 30°C, 35°C, 40°C and 45°C. Yield of uricase was studied in these temperatures range along with the respective biomass.

Salinity (NaCl concentration)

Different salinity ranging from 0.5 – 3% (at the interval of 0.5%) were maintained in the medium and incubated.

Carbon sources

Various carbon sources used in the medium were glucose, fructose, sucrose, lactose, maltose, cellulose and starch were used at a concentration that replacing the C source in the Czapek-Dox agar medium.

Optimization of carbon source concentration

In the medium, the ideal carbon source (sucrose) was added at varied concentration. Various concentration of the carbon source kept for optimization study were 5%, 10%, 15%, 20% and 25% (w/v).

Nitrogen Sources

Nitrogen sources used in the medium were peptone, yeast extract, beef extract, casein, uric acid, $NaNO_3$, NH_4NO_3, KH_2PO_4 and Na_2HPO_4 at a concentration of 0.3%.

Optimization of concentration of nitrogen source

Concentration of nitrogen source was kept varied from 0.2, 0.3, 0.4, 0.5 and 0.6% (w/v).

Effect of different cheaper substrates

Different substrates such as, used waste motor oil, molasses, whey, poultry waste at 1% concentration was tried.

Mass scale culture in shake flask and fermentor

Using all the optimized parameters (i.e.) Incubation time – 96hrs, Agitation speed – 150 rpm, pH - 8, Temperature – 30°C, Salinity – 2%, sucrose – 10% (w/v), uric acid - 0.4% (w/v), Mass scale culture was done in shake flask. Poultry waste (1%) was separately tried as a cheaper substrate for mass scale production. The parameters optimized for shake flasks were used for mass scale production in a 3L lab scale fermentor DO (Dissolved oxygen) was maintained at 60% using air compressor based on trial runs in the fermentor. Biomass and enzyme activity were assessed.

Enzyme assay

Uricase activity was measured according to the procedure described by Adamek *et al.*, 1989. To 2ml of a solution containing uric acid (10µg per ml of borate buffer 0.2M, pH 8.5), 0.8ml of water and 0.1ml of crude enzyme at 25°C were added. After 10 min. 0.2ml of 0.1M potassium cyanide solution was added to the mixture to stop the enzyme reaction. The absorbance of samples was measured at 293nm. The difference between absorbance of the sample and reference is equivalent to the decrease in uric acid during the enzyme reaction. One unit of uricase enzyme was equal to the amount of enzyme which converts 1µmol of uric acid to allantoin per min. at 30°C.

Results and Discussion

In the present study sediment samples were collected from Pichavaram mangrove area. The fungal species were isolated by serial dilution plating method in Czapek-Dox medium (Fig. 2). Screening of fungal isolates for uricase production was performed (Table – 1 and 2). The most potential strain was optimized for uricase production. It was identified as *Aspergillus niger* using standard manuals (Figs. 3 and 4).

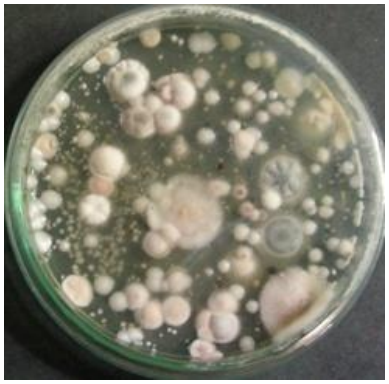

Fig. 2: Isolation of fungi from mangrove sediment on Czapek-Dox agar plate

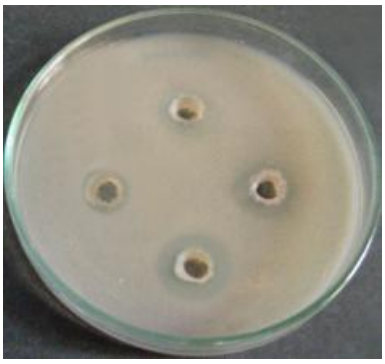

Fig. 3: Plate showing uricolytic activity

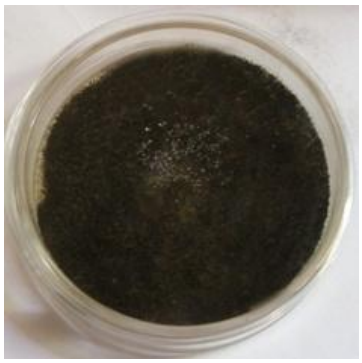

Fig. 4: *A. niger* on Czapek-Dox agar plate

Fig. 5: Lactophenol cotton blue staining and light microscopic observation of *A. niger* (400X)

Table – 1: Uricolytic activity of fungal strains isolated from mangrove sediment by well diffusion assay

Strain No.	Zone of Clearance (diameter in mm)
Alternaria alternata	10
Rhizoctonia solani	11
Aspergillus niger	16
Aspergillus flavus	9
Fusarium oxysporum	-
Penicillium citrinum	12

Table 2: Screening of fungal isolates for uricase production

	Alternaria alternata	*Rhizoctonia solani*	*Aspergillus niger*	*Aspergillus flavus*	*Fusarium oxysporum*	*Penicillium citrinum*
Biomass	4.3	3.7	6.4	5	1.8	2
Uricase activity (Extracellular)	88	67	148	97	21	17
Uricase activity (Intracellular)	23	14	-	-	2	6

Compared to bacteria only few works are available on fungal uricase production. However the work started as early as 1937. Geweely and Nawas (2011) observed both intracellular and extracellular enzymes in *A.niger* whereas in the present study *Aspergillus* sp used did not produce any intracellular enzyme. Watanabe and Fukumoto (1970) studied the production of uricase in *Streptomyces*. They found that uricase production was induced in resting cells.

Optimization is as important as growth and production of metabolites which is strain dependent. Hence optimization was done in the present investigation for the following parameters (Figs. 6-15). El-Dein and El-Fallal (1996) found the highest uricase producer *A. carbonarius* produced only 0.16U/ml/min in screening medium. *A. sydowi*, *A. terreus*, *A. niger* and *A. alutaceus* produced below 0.06U/ml/min. and the least producer was *A. alutaceus*.

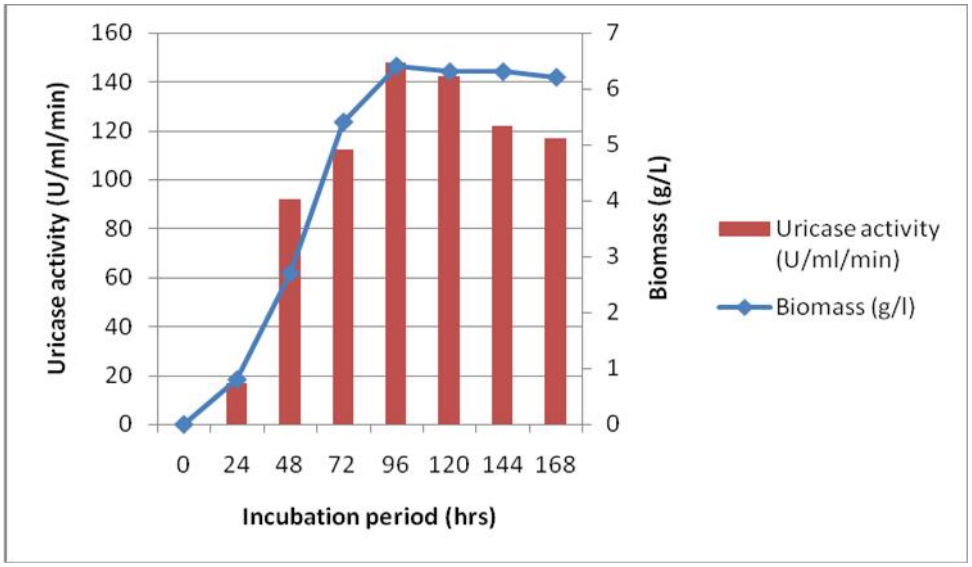

Fig. 6: Effect of incubation period on biomass and uricase production in *A. niger*

Aspergillus niger spores were inoculated in the medium and kept in different agitation speed to achieve higher uricase and biomass production. The different agitation speeds were 50 rpm, 100 rpm, 150rpm and 200rpm. A flask was maintained at 0 rpm (at static condition). All the flasks were incubated for 96hrs. A maximum of biomass and uricase at 96hrs of incubation was 6.3 g/L and 141 U/ml/min. respectively. The minimum growth was observed with 0 rpm (i.e.) at static condition 2.2g/L of biomass was obtained whereas the uricase activity was 44U/ml/min. (Fig.7). Compare to static condition, agitation resulted in better growth. Aly *et al.*, 2013 also observed 100 rpm as an ideal for *Streptomyces exofoliatus* in uricase production.

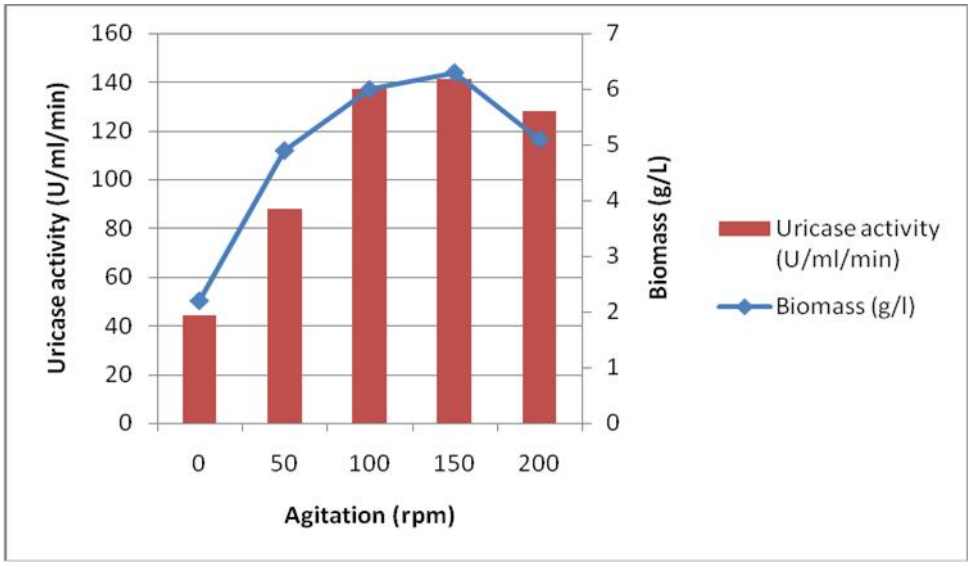

Fig. 7: Effect of agitation on biomass and uricase production in *A. niger*

In the present study 4, 5, 6, 7, 8, 9, 10, 11 and 12 were tested, and the maximum biomass and production of uricase was observed at pH-8 (i.e.) (6.2g/L) and 143 U/ml/min. respectively The minimum growth was observed with pH-11 (2.11g/L) where the uricase activity was 12U/ml/min. (Fig.8). Li *et al.*, 2006 also found pH 8 as optimum for the *A. flavus* when its uricase gene was expressed in *E.coli.* Aly *et al.*, 2013 found maximum production at pH 6.5 which decreased significantly at pH 9 in *Streptomyces exfoliatus* UR 10 strain isolated from farm waste.

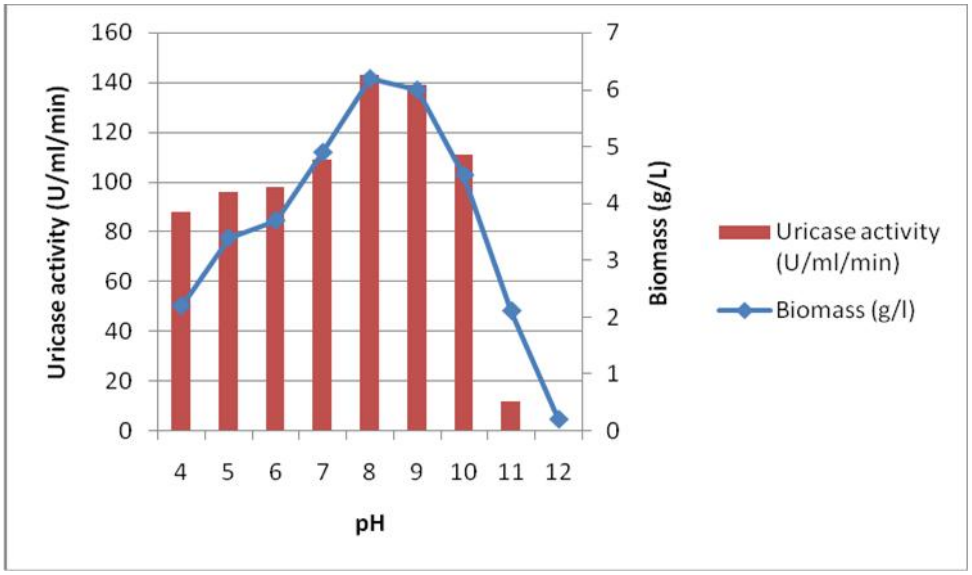

Fig. 8: Effect of pH on biomass and uricase production in *A. niger*

Regarding temperature range of 20°C – 45°C at an interval of 5°C were tested. Maximum biomass and uricase production were 5.8 g/L and 140 U/ml/min. and the minimum growth was and uricase activity was observed at 45°C (1.17g/L) and 21U/ml/min. respectively (Fig.9).

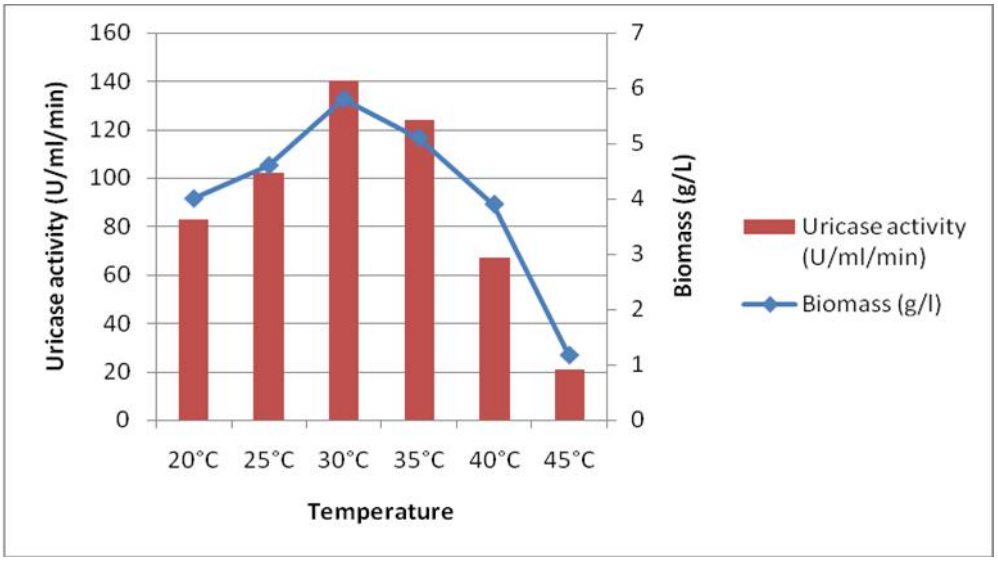

Fig. 9: Effect of temperature on biomass and uricase production in *A. niger*

Different salinity ranging from 0.5 – 3% (at the interval of 0.5%) were maintained in the medium and incubated at 30°C for 96hrs. 2% salinity favoured maximum biomass and uricae enzyme production respectively of 6.3g/L and 140U/ml/min. The minimum biomass was observed with 0.5% NaCl (2.1g/L) where the uricase activity was 67U/ml/min. (Fig. 10)

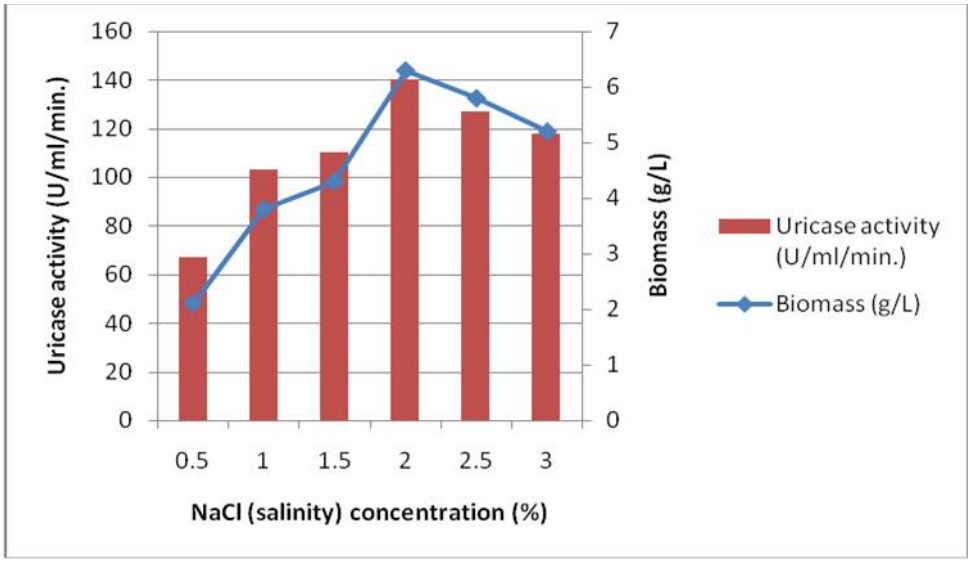

Fig. 10: Effect of NaCl concentration (salinity) on biomass and uricase production in *A.niger*

In the present, study sucrose was found to be the best C source for uricase production where 6.1g/l and 144U/ml/min of uricase production was observed. The minimum biomass was observed with starch (1.9g/L), where the uricase activity found was 68U/ml/min. (Fig.11). Regarding the concentration of the most potent carbon source, sucrose at 10% concentration favoured 6g/L and 141 U/ml/min of biomass and uricase respectively. Increased concentration leads to reduction in both growth as well as enzyme activity. At 25% sucrose the minimum growth of 3.4g/L with an uricase activity of 98U/ml/min. were observed (Fig.12). Hatijah and Ruhayu (2013) recorded sucrose (30%) as the ideal c source for uricase production in *A.flavus.(*i.e).*A.niger* strain used in the presen study perfered lower concentration of sucrose compared to *A.flavus*. Anderson and Vijayakumar (2011) found maltose as the best carbon source. Abbas (2016) recorded lactose as the potent carbon source.

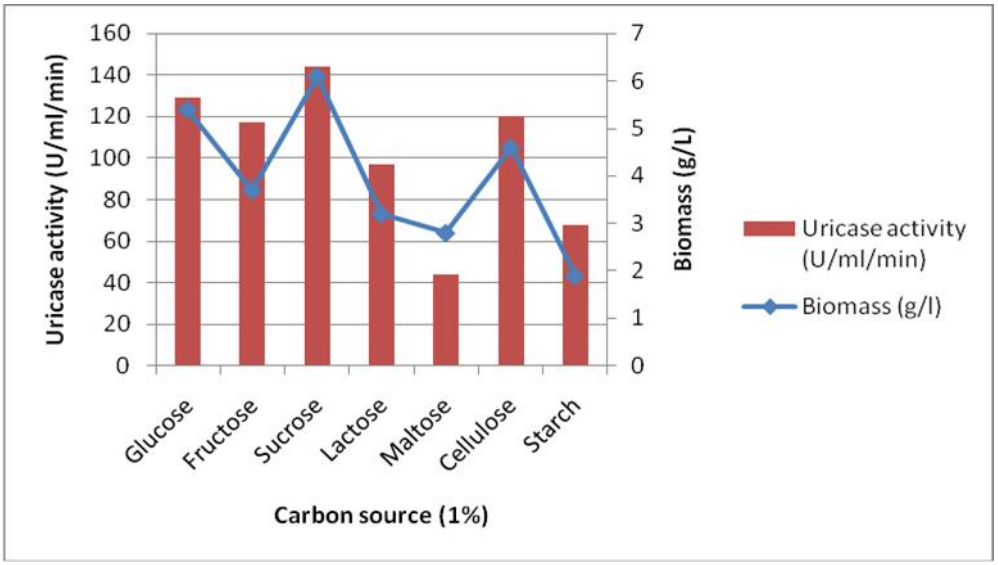

Fig. 11: Effect of carbon source on biomass and uricase production in *A. niger*

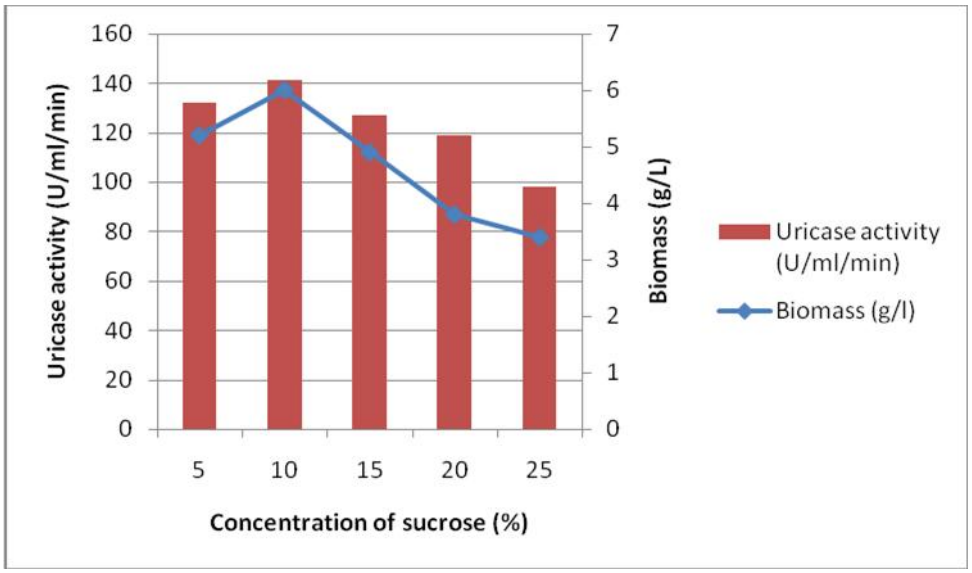

Fig. 12: Effect of concentration of carbon source (sucrose) on biomass and uricase production in *A. niger*

Regarding the nitrogen source uric acid as a nitrogen source resulted in maximum biomass (6.4g/L) and uricase activity (146 U/ml/min.). The minimum biomass was observed with $NaNO_3$ (2.1g/L) where the uricase activity was 68U/ml/min. (Fig. 13). *Aspergillus alutaceus* and *Penicillium oxalicum* the weakly positive fungi, grew well in presence of uric acid. In a *Streptomyces exfoliates* UR10 strain 0.2% uric acid acted as an inducer for uricase enzyme production (Aly *et.al.*, 2013). Nanda *et al.*, 2012 found that peptone (12.71g/L) and yeast extract (10.57g/L) enhanced uricase activity in *Gliocladium viridae*, a fungal strain, casein and peptone were observed as the best nitrogen sources resulting in 4.86 and 3.16U/ml of uricase in *A. niger* (Ali and Ibrahim, 2013). The concentration of nitrogen source, uric acid was tested from 0.1-0.6% where 0.4% concentration favored a maximum biomass of 5.9g/L and uricase activity of 140 U/ml/min. The minimum biomass was observed with 0.1% uric acid (1.9g/L), where the uricase activity was observed as 87U/ml/min. (Fig. 14).

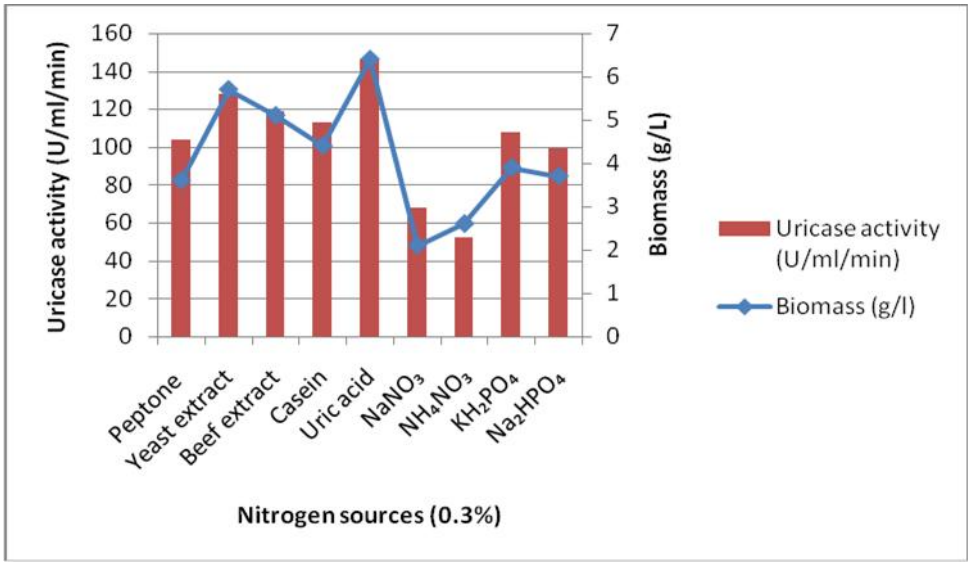

Fig. 13: Effect of nitrogen source on biomass and uricase production in *A. niger*

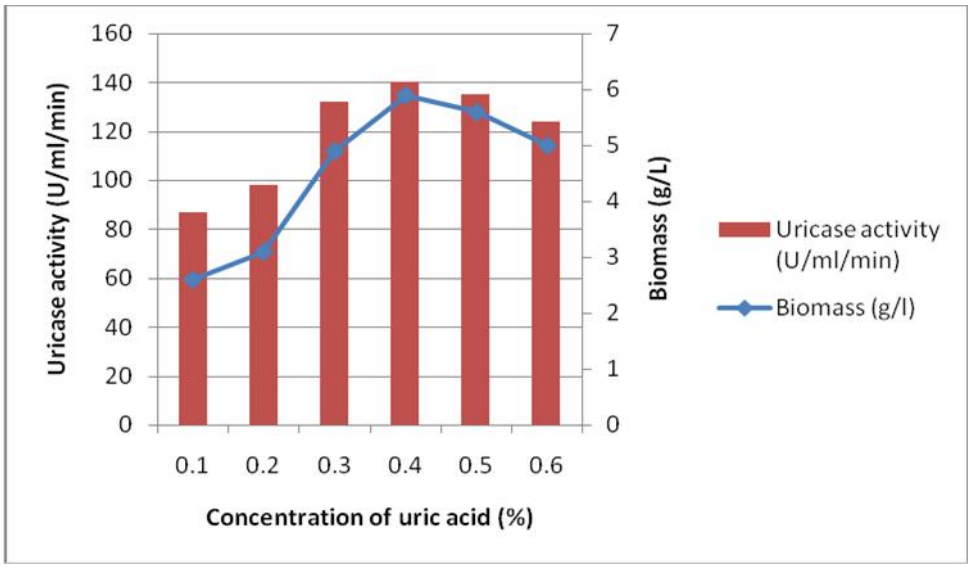

Fig. 14: Effect of concentration of nitrogen source (uric acid) on biomass and uricase production in *A. niger*

Different substrates such as, used motor oil, molasses, whey, poultry waste at 1% concentration were tried in which 1% poultry waste favored maximum biomass of 6.7g/L with 135U/ml/min of uricase activity respectively. The minimum growth was observed with used waste motor oil (3.6g/L) where the uricase activity observed was 88U/ml/min. (Fig. 15).

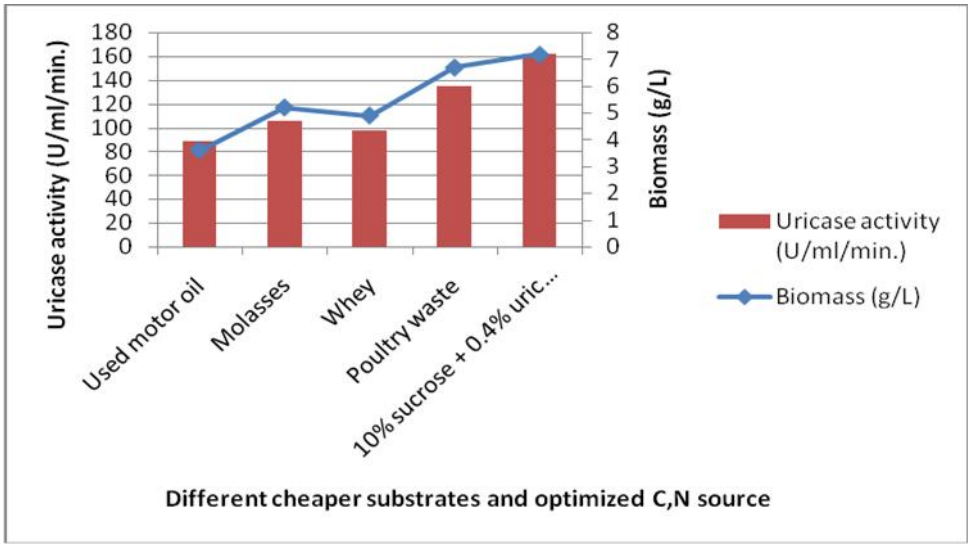

Fig. 15: Effect of different cheaper substrate on biomass and uricase production in *A. niger*

All the optimized parameters (i.e.) Incubation time – 96hrs, Agitation speed – 150 rpm, pH - 8, Temperature – 30°C, Salinity – 2%, sucrose – 10% (w/v), uric acid - 0.4% (w/v) were kept for mass scale production and the estimation of uricase was performed as previously. Poultry waste was separately tried as a cheaper substrate for mass scale production. In the present study the maximum biomass and uricase production was observed in mass scale 7.18g/L and 162U/ml/min. in shake flasks with standard substrate. It was 6.7g/L and 135U/ml/min. in shake flasks with cheaper substrate. Similarly in fermentor the biomass and enzyme production using standard and cheaper substrates were 9.6g/L and 232U/ml/min. and 8.4g/L and 189U/ml/min. respectively (Fig. 16). The study clearly indicated the potential of *A. niger* strain of mangrove sediment origin for uricase production.

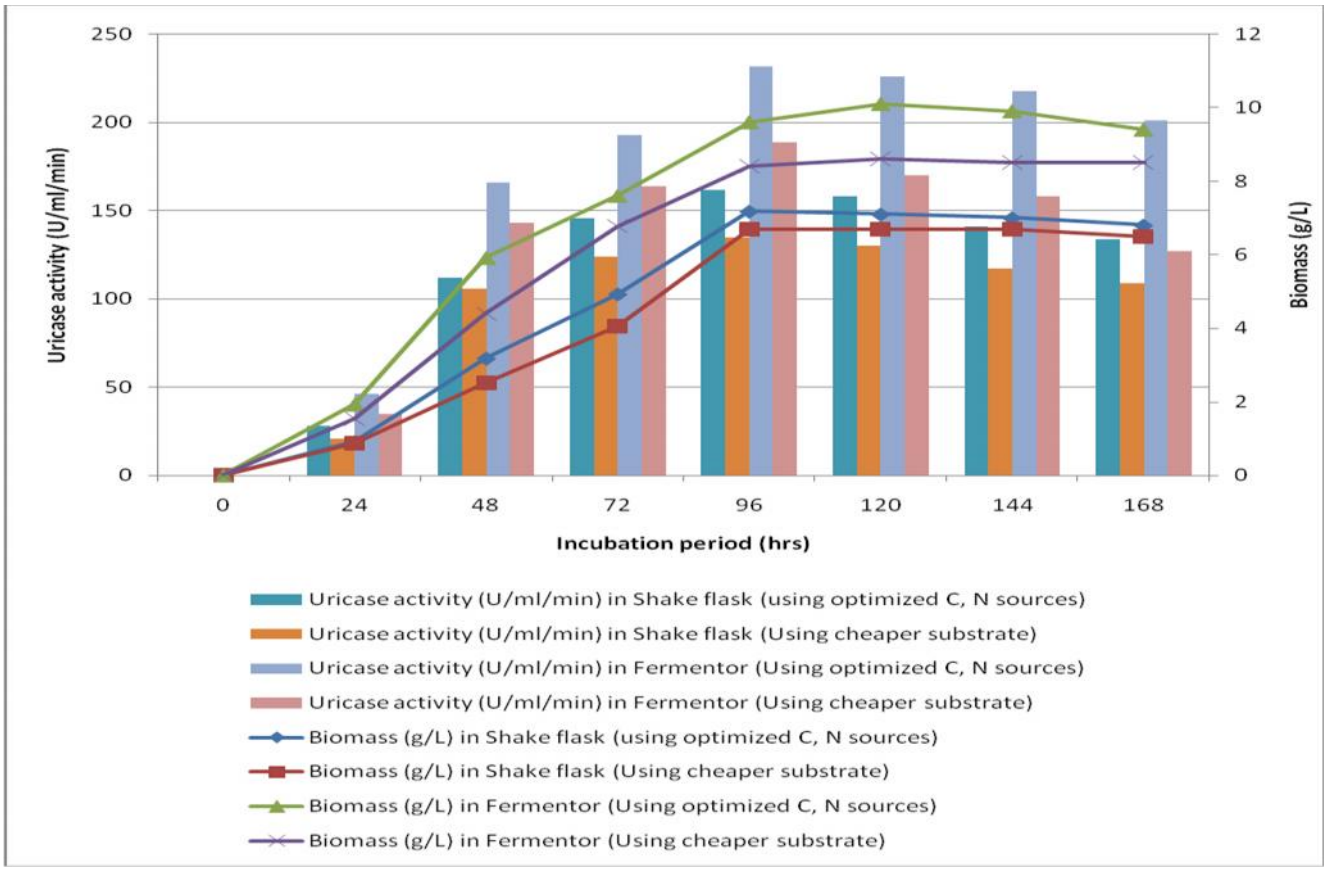

Fig. 16: Mass scale *A.niger* biomass and uricase production

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