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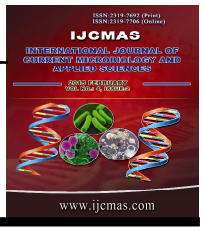

Original Research Article

Screening and Optimization of Organic acid Producers from Mine Areas of Chhattisgarh Region, India

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Keywords

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A B S T R A C T

Solubilization of metals by microorganisms can be exploited for effective metal recovery from low grade ores as the chemical leaching techniques are not cost effective. Fungi are able to solubilize metals and metalloids from insoluble compounds such as ores, metal phosphates, sulfides and oxides by chelating metal ions. Mine areas are known for acidic environment due to the oxidation of pyrite and other sulfide minerals. The main objective of this work was to isolate and identify the fungal species from mines areas of Chattisgarh region capable of producing larger quantity of organic acids for solubilization of metals. A total of twenty nine acidophilic fungi were isolated from mine area of Chhattisgarh. They were later analyzed for acid production both qualitatively and quantitatively. Four isolates belonging to the genera *Aspergillus* and *Penicillium* produced acid significantly. In the present study optimization of one best producer *viz*., *Aspergillus niger* gr.NFCCI-3303 with different parameters using one factor at a time (OFAT) method was employed to find out the possible optimum carbon, nitrogen, inoculum size and incubation period. 5% Glucose, 0.3% urea, 4mm inoculum size and 25 days of incubation period was found to be optimum for maximum acid production.

Introduction

The fungi as a group contain widely diverse type of microorganisms, ranging in size from microscopic to the easily visible mushrooms and puffballs. Their metabolic activities are similarly diverse, and literally hundreds of products are formed by the group. Many of these products are acids; review lists forty-one known acids among which only few are of interest *viz*., citric, gluconic, fumaric, gallic, *d*-lactic, itaconic, kojic and oxalic acids (Cochrane, 1948). Organic acids possess a long chain of carbons attached to a carboxylic group. This category of acid is used widely in the food industry. Citric acid is the most widely used acidulant in the food and beverage industries, it has many uses in many forms, examples including as a blood anticoagulant,

metal cleaning agent and a de-scaler. Fermentation processes play a major role in the production of most organic acids. All acids of the tricarboxylic acid (TCA) cycle can be produced microbially in high yields; other acids can be derived indirectly from the Krebs cycle such as itaconic acid, or can be derived directly from glucose (gluconic acid). Some acids are formed as the end products from pyruvate or ethanol (lactic and acetic acid). Fungi, in particular *Apergilli*, are well known for their potential to overproduce a variety of organic acids. These microorganisms have an intrinsic ability to accumulate these substances and it is this that provides the fungi with an ecological advantage, since they grow rather well at pH 3 to 5, while some species even tolerate pH values as low as 1.5.

The biochemistry of the production of citric acid by *A. niger* involves two major steps, (a) the breakdown of the carbohydrate source to pyruvate and acetyl CoA, (b) the formation of oxaloacetate from pyruvte and CO_2. This leads to the accumulation of citric acid in the TCA cycle. Thus far microbes have mainly been used for the production of antibiotics, enzymes and organic acids for commercial application, however, their ability to solubilize metals from solid material has opened up completely new prospects for their application in mineral biotechnology for metal leaching from low grade ores (Joshi and Luthra, 2000).

The fungus *A. niger* has been found to produce organic acids that can serve as leaching agents for the solubilization of metals (Bosshard *et al*., 1996). Bioleaching with fungal microorganisms is based on the following mechanisms: solubilization of the matrix, complexation of metals by the excreted organic acids or amino acids, reduction of ferric iron which is mediated by oxalic acid, and bioaccumulation of metals by the organism´s mycelium. Microbes such as bacteria and fungi convert metal compounds into their water-soluble forms and are biocatalysts of these leaching processes (Acharya, 1990). Solubilization of metals from ores by fungi is well documented by many researches (Rekha *et al*., 2001, Hefnawy *et al*., 2002, Rao *et al*., 2002, Mulligan and Kamali, 2003, Acharya *et al*., 2004, Bohidar *et al*., 2009; Behera *et al*., 2011).The main objective of this work is to screen acidophilic heterotrophic fungus capable of producing organic acids form oxidation of carbon source by its metabolic activity and also to optimize the fermentation process using one factor at a time (OFAT) method.

Material and Methods

Study Area

Samples were aseptically collected from the vicinity of soils from seven mine areas of Chhattisgarh region *viz*., Dalli Rajhara Mine, Bailadila Mine, Ahiwara Mine, Rawan Mine, Hirri Mine, Korba Mine and primary sites of Bhilai Steel Plant.

Sample Collection

Samples were collected using aseptic zipped polythene bags and were transported to the laboratory and stored at 4^0C in the refrigerator till use.

Isolation of Fungi

Fungus was isolated by serial dilution agar plating method on Potato dextrose agar plates in triplicates. Based on predominance and distinct morphological properties fungal colonies were selected and purified by repeated subculturing and streak plating. The microorganisms were sub cultured once in a month.

Screening for organic acid production

Qualitative acid production assay

The fungal isolates were subjected for qualitative assay for acid production using acid indicator medium (AIM) containing 0.04% of bromocresol purple (Das and Roy, 1998).

A loopful of fungal spore was inoculated on Czapek-Dox broth medium contained (g/l) : Sodium nitrate 2.0, Dipotassium hydrogen phosphate 1.0, Magnesium sulphate 0.5, Potasssium chloride 0.5, Ferrous sulphate 0.01, sucrose 30, bromocresol purple 0.04 in distilled water and incubated for five days for the formation of yellow coloration of the medium which indicated production of acid.

Quantitative acid production assay

The positive fungal isolates were further assayed for quantitative acid production. The total acidity of the culture filtrates was estimated by titration method, by taking 10ml of fermented broth against 0.1N NaOH (standard alkaline solution) using phenolphthalein as indicator (Peppler, 1967; El-Ktatney, 1978) and strength of acid production was calculated in terms of molarity (M). The isolates showing greatest acid production were selected for further study.

Identification of Fungal Isolates

The fungal isolate with significant level of organic acid production was streaked aseptically on Potato dextrose agar plates incubated at 28 ± 2^0 C for five day and assessed for its morphological properties. Further it was characterized through National Fungal Culture Collection of India (NFCCI), Pune.

Results and Discussion

A total of 29 acidophilic fungal isolates were isolated from different mine areas of Chhattisgarh Region which exhibited acid production ability both qualitatively and quantitatively (Fig. 1). Five isolates LAK-2, BS-1.6, CM-2, DR*-1 and DR-2 showed good acid production. Table 1 describes the identification of five fungal isolates with their accession number *viz*., LAK-2 as *Aspergillus niger gr.* (NFCCI-3303), DR-2 as *Trichoderma atroviride* Karsten (NFCCI-3304), BS-1.6 as *Penicillium* sp1 (NFCCI-3305), CM-2 as *Penicillium* sp2 (NFCCI-3306) and DR*-1 *Aspergillus niger gr.* (NFCCI-3307) respectively identified by National Fungal Culture Collection of India, Pune. LAK-2 (*Aspergillus niger gr.*) strain was further selected for optimization process using one factor at a time (OFAT) to determine optimum fermentation conditions. To evaluate the most significant medium constituents affecting acid production four factors *viz*., carbon, nitrogen, inoculum size and incubation period were initially chosen to find out the possible carbon and nitrogen source and optimum level of concentration of the above selected parameters and result was analyzed via one way ANOVA by SPSS (Version 16.0). A medium of the composition (g/l): sodium nitrate 2.0, dipotassium hydrogen phosphate 1.0, magnesium sulphate 0.5, potassium chloride 0.5, ferrous sulphate 0.01 and Glucose 30 (pH 7.3) was used as the control. To study the effect of carbon sources on the acid production, six carbon sources i.e. sucrose, maltose, lactose, fructose, xylose and starch were substituted in place of glucose in the culture medium and acid production was assayed. The results of fermentation using different carbon compounds are summarized in Figure 2. It was found that the acid production of *Aspergillus niger gr.* (LAK-2) was maximum with glucose, the value being

0.12±0.01M followed by sucrose, starch, maltose, xylose and fructose whereas the minimum level of production was recorded with lactose, the value being 0.01±.002M (p= 0.0001).

The concentration of carbon sources plays an important role on the conversion of glucose into acid (Shindia *et al*., 2006). Different glucose concentrations ranging from 3, 5, 10 and 15 percentages were investigated to see the effect of carbon level on this fermentation reaction (Fig. 3). In *A. niger* the lowest level of acid production was recorded in the presence of 3% glucose, the value being 0.14±0.01M, the production level started increasing slightly with increase of glucose concentration and maximum level was recorded at 10%, the value being 0.19±0.006M. Further increase in glucose concentration, the production decreased to 0.16±0.013M according to Kovats (1960) found initial sugar concentration critical for citric acid production and other organic acids produced by *Aspergillus niger*. Xu *et al*. (1989) reported that *A. niger* strains needed an initial sugar concentration of 10–14% as optimal; no citric acid was produced at sugar concentration of less than 2.5%. The acid production is directly influenced by the nitrogen source. Physiologically, ammonium salts are preferred, e.g. ammonium sulfate, ammonium chloride, peptone, malt extract etc. Nitrogen consumption leads to pH decrease, which is very important in citric acid production fermentation (Rohr *et al*., 1983, Kubicek and Rohr, 1986).

To select the most appropriate nitrogen source, nine different organic and inorganic nitrogen sources i.e. ammonium sulphate, ammonium chloride, ammonium nitrate, potassium nitrate, urea, yeast extract, peptone, beef extract and malt extract were substituted in place of sodium nitrate in the culture medium (Fig. 4). The highest level of acid production was found in the presence of urea, the value being 0.19±0.014M whereas the lowest level of production was observed in the presence of malt extract, the value was 0.05±0.004M. Since urea was found to be the best nitrogen source, different concentration of urea was used to observe the higher production of acid ranging from 0.2 to 0.6 percent. *Aspergillus niger gr*. exhibited the highest level of acid production with 0.3% urea, the value being 0.25±0.011M followed by 0.2% and 0.4%, the value being 0.19±0.016M and 0.20±0.013M respectively the value decreases with increase in urea concentration (Fig. 5). Urea has a tampon effect, which assures pH control (Raimbault, 1980). A high nitrogen concentration increases fungal growth and the consumption of sugars, but decreases the amount of citric acid produced (Hang *et al*., 1977).

To check the efficacy of inoculum size on acid production by fungal strain three sizes *viz*., 6mm, 8mm, 10mm were substituted in place of 4mm inoculum size. No significant difference in acid production was found by varying the inoculum sizes (Fig. 6). Acid production values steadily increased with fermentation time (Kareem *et al*., 2010). Acid production significantly increased with increasing the incubation period (Fig. 7) and production was observed up to 30 days of incubation period, which was found to be maximum on the 25 days of incubation period *i.e.*, 0.59±0.25M which further increase the time, the production level decreases with 0.39±0.022M.

In conclusion, from these findings it becomes clearly indicated that for higher productivity of acids, optimization of process variable is required. The present study includes the parameters: carbon,

nitrogen, inoculum sizes and incubation time which were tested for the production of acids. Acid production was found to be highly and positively influenced by both the carbon and nitrogen sources which further enhances by varying the initial concentration of both the parameters. Changing the inoculum sizes does not cause any significant change in acid production where as there is a strong impact on acid production with increase in incubation period. One factor at a time (OFAT) offers the possible finding of the parameters for optimization process, though it is not of first priority in the screening program to examine the interaction between the variables. *Aspergillus niger gr.* (LAK-2) showed the positive influence on acid production by varying the above parameters. However in the present study specific acids were not identified since the study was only concerned about the production of total acids which were in turn help in bioleaching study as total acid is related to leaching of metals.

Table.1 Identification of Fungus based on morphological characters by NFCCI

Sr.	Culture	NFCCI Accession	Identification Remarks
1.	LAK-2	NFCCI-3303	*Aspergillus niger gr.*
2.	DR-2	NFCCI-3304	*Trichoderma atroviride* Karsten
3.	BS-1.6	NFCCI-3305	*Penicillium* sp 1
4.	CM-2	NFCCI-3306	*Penicillium* sp 2
5.	DR*1	NFCCI-3307	*Aspergillus niger gr.*

Fig.1 Quantitative acid production of twenty nine fungal isolates

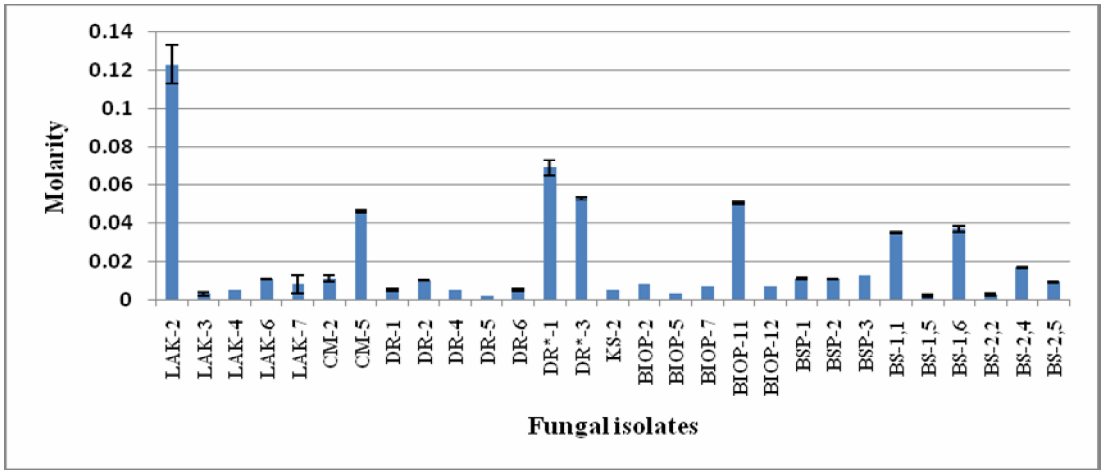


Fig.2 Effect of carbon sources on acid production of *Aspergillus niger gr.* (LAK-2)

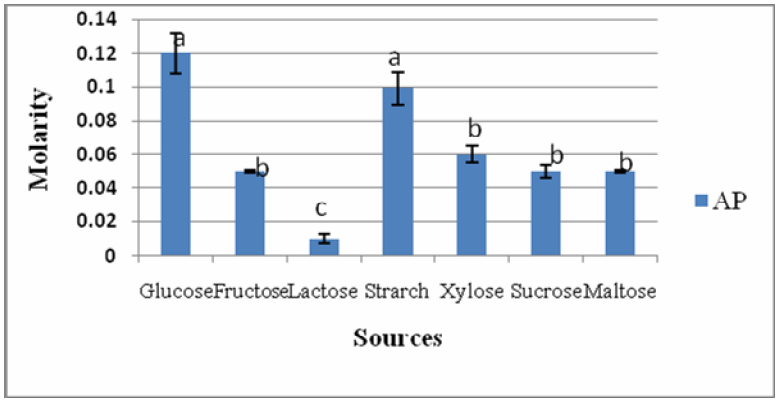


Fig.3 Effect of glucose concentration on acid production of *Aspergillus niger gr.* (LAK-2)

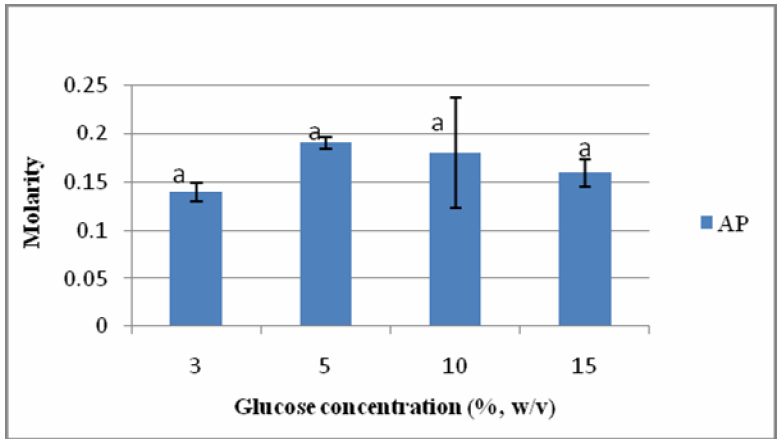


Fig.4 Effect of nitrogen sources on acid production of *Aspergillus niger gr.* (LAK-2)

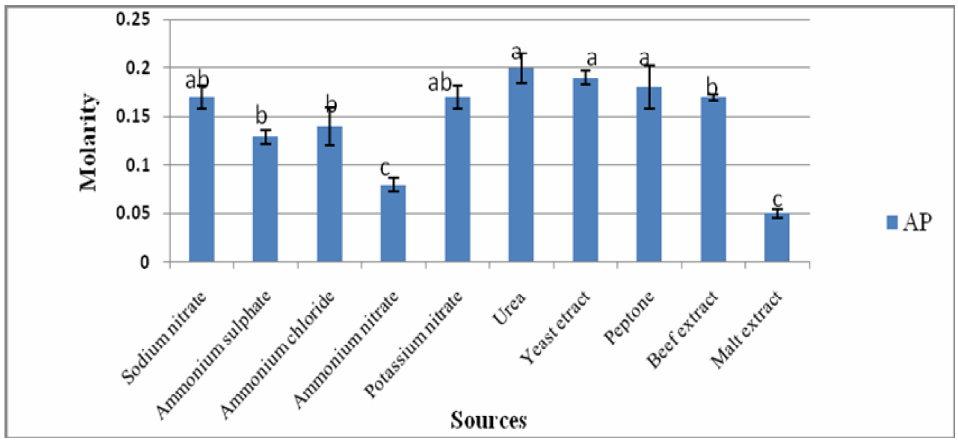


Fig.5 Effect of urea concentration on acid production of *Aspergillus niger gr.* (LAK-2)

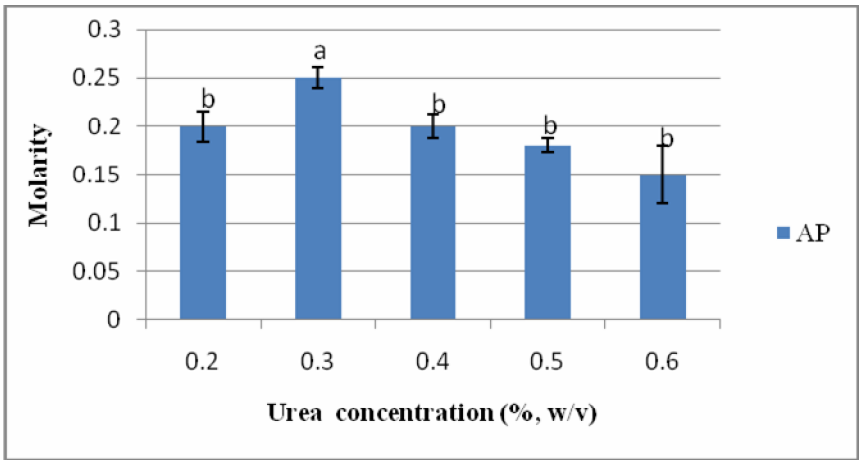


Fig.6 Effect of inoculum sizes on acid production of *Aspergillus niger gr.* (LAK-2)

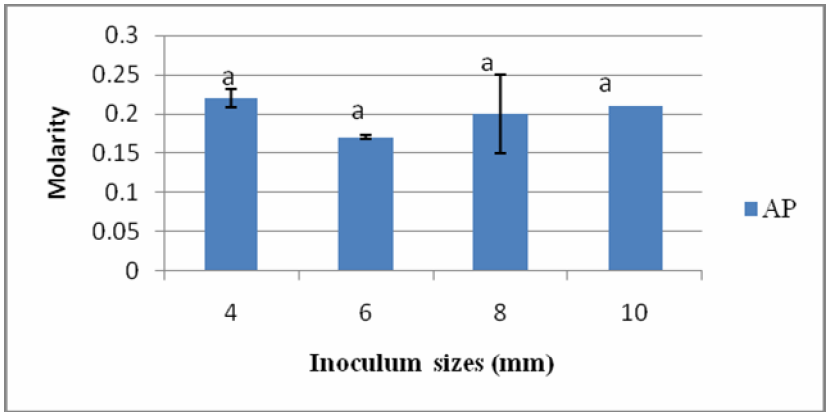
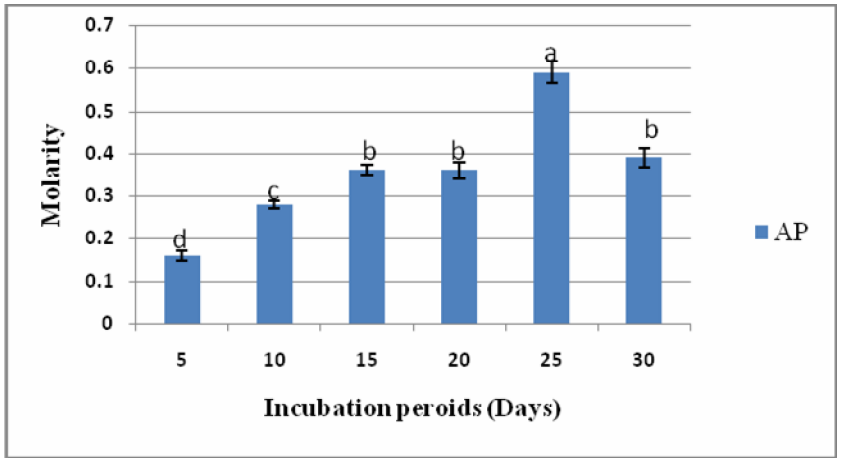


Fig.7 Effect of incubation period on acid production of *Aspergillus niger gr.* (LAK-2)



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