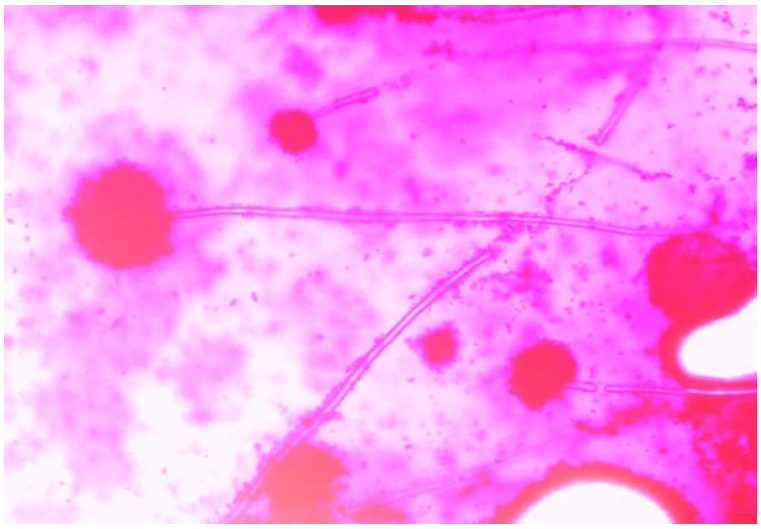
ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

SMALL-SCALE CITRIC ACID PRODUCTION ON SOLID-STATE FERMENTATION USING ASPERGILLUS NIGER



BY

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Dedicated to

My brother Mehabaw Feleke

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ABREVATION

ASAS	*Aspergillus niger* strain from air sample
ASBS	*Aspergillus niger* strain from barely seed
ASCS	*Aspergillus niger* strain from coffee seed
ASGS	*Aspergillus niger* strain from green house soil
ASMS	*Aspergillus niger* strain from Maize growing soil
ATP	Adenosine Tri phosphate
aw	Water activity
CA	Citric acid
CB	Cassava bagasse
CZA	Czapek Dox Agar
DR	Dry Rice
DS	Dry Substrate
ECSA	Ethiopian Central Statistics Agency
g/l	gram per liter
hr	hour
ICA	Isocitric acid
kJ	Kilo jule
kg	Kilogram
LPAB	Lacto Phenol Aniline Blue
M	Shredded maize
min.	minute
ml	mililiter
mμ	mili micro

µgm	microgram
MR	Combination of shredded maize and rice
MW	Combination of shredded maize and wheat bran
MWR	Combination of shredded maize, wheat bran and rice
NADPH	Nicotinamine adenine diphosphate
O.D.	Optical Density
PDA	Potato Dextrose Agar
R	Rice
rpm	revolution per minute
SLF	Submerged Liquid Fermentation
SSF	Solid State Fermentation
TCA	Tri carboxylic acid cycle
v/w	volume by weight
W	wheat bran
WR	Combination of wheat bran and rice
w/w	weight by weight

ABSTRACT

Citric acid is used in the food, beverage, pharmaceutical, chemical, cosmetic and other industries for applications such as acidulation, anti oxidation, flavour enhancement, preservation, and as a synergistic agent. It is used to flavour the drinks, jams and jellies, candies, water ice and wines. In recent years, a great deal of attention is given to alternative methods for citric acid production using agro-industrial residues. A citric acid producing *Aspergillus niger* isolate designated as ASGS was isolated from the Faculty of science Addis Ababa University green house soil. The isolate produced citric acid in solid state fermentation (SSF) using different agricultural products and by products (Maize, Rice and wheat bran) as substrates. Out of the three substrates, rice(R) was found to be better followed by the combination of wheat bran and rice (WR). The highest level of citric acid (110.6g/kg DR) was produced in Rice, with Rice-to- moisture ratio of 1:4 (w/v) at 30ºC and 72 hr. The weakest substrate for citrate fermentation was wheat bran with only 7g/kg citrate production. Maximum growth of the isolate was shown at 30°c, it also showed growth and biomass accumulation at 35°c. Wrinkled, dense, white mycelium was observed in the flask at 30°c while at 25°c was not shown. Citrate accumulation increased exponentially after 48hrs of fermentation and the maximum citric acid (82.6g/kg DR) was obtained at 72hr which declined gradually. Initial rice moisture content had a considerable effect on citric acid production by ASGS. A maximum citric acid production (91g/kg DR) was obtained with 80% moisture content. Citrate accumulation also increased significantly with increase in temperature from 25 to 30°c and production slightly declined up to 35°c. The highest value of citric acid concentration (98g/kg DR) was achieved at an initial pH of 4.5. Recovery trial yielded 1.2g citric acid from 10g of dry rice substrate which was white and solid crystalline.

Key phrases: *Aspergillus niger strain; Solid state fermentation; Citric acid.*

1. INTRODUCTION

Citric acid is a tricarboxylic acid with a molecular weight for anhydrous and monohydrate is 192 and 210g/mol respectively. It is a nearly universal intermediate product of metabolism and its traces are found in virtually all plants and animals (Papagianni, 2007). It is solid at room temperature melts at 153 °C and decomposes at higher temperature (Kubicek, 1998).

Among the organic acids industrially produced, citric acid is the most important in quantitative terms. Citric acid is used in the food, beverage, pharmaceutical, chemical, cosmetic and other industries for applications such as acidulation, anti oxidation, flavour enhancement, preservation, and as a synergistic agent (Sarangbin *et al*., 1993). It is used to flavour the drinks, jams and jellies, candies, water ice and wines (Archer, 2000). The food industry consumes about 70 % of total citric acid produced and pharmaceutical industries consume about 12 %, and the remaining 18 % are consumed by other industries (Pandey *et al*., 2001; Soccol *et al*., 2003). Global production has now reached 1.4 million tones and there is annual growth of 3.5–4.0 % in demand/consumption of citric acid (Soccol *et al*., 2006).

Citric acid production can be under taken by different groups of microorganisms. Although yeast, bacteria and other *Aspergillus* species can produce citric acid, *A. niger* remained the organism of choice for commercial production, because it produces more citric acid per time unit (Soccol *et al*., 2006).

The problem in the production of citric acid by yeasts is the simultaneous formation of isocitrate. The main advantages of using *Aspergillus niger* are its ease of handling, its ability to ferment a variety of cheap raw materials and high yields (Soccol *et al*., 2006). Recently, a wide range of citric acid production has been reported in response to different levels of nutrient supplementation (Bari *et al*., 2009). *A. niger* is capable of producing very high levels of citric acid, about 90% of the theoretical yield from a carbohydrate source (Kubicek and Rohr, 1986).

A high rate of acidogenesis in *A. niger* is observed only under conditions of high glycolytic metabolism and can be induced by the addition of an excess amount of sucrose or other carbohydrates. Citric acid accumulation is known to be stimulated by high sugar concentration suggesting it may be influenced by osmotic stress (Peksel and Kubicek, 2001).

The production of citric acid using cheap carbon source from agro-industrial byproducts provides considerable advantage combined in the benefit of waste management as well as decrease in the cost of citric acid production (Kumar *et al*., 2003; John *et al*., 2006). The use of alcohols as a stimulant in citric acid production may enhance the yield of citric acid (Wieczorek and Braver, 1998). The stimulatory effect of ethanol and methanol on citric acid production can be explained in terms of mycelia morphology as well as pellet shape and size (Barrington, 2008). Citric acid production could be increased by exploiting available resources and adding the stimulatory agents to the fermentation medium (Pera and Callieri, 1997).

Citrate accumulation in substantial amounts depends on certain environmental parameters. It is accumulated when several nutrient factors are present (Yigitoglu, 1992). One of these factors is the concentration and type of sugar (Hossain, *et al*., 1984). The production of citric acid also depends strongly on an appropriate strain and on operational conditions such as aeration, type and concentration of the carbon source, nitrogen and phosphate limitation, pH, concentration of trace elements and the morphology of the producer organism (Papagianni, 2007).

To date, most industrial processes are carried out with submerged fermentation of *Aspergillus niger*. Although citric acid can be obtained by chemical synthesis, the cost is much higher than using fermentation. It is mainly produced by submerged fermentation by the filamentous fungus *Aspergillus niger*. *A. niger* has been used commercially for the first time in 1923 for citric acid production. In order to decrease the cost of citric acid production using *A. niger*, solid state fermentation has been studied as a potential alternative to submerged fermentation.

Solid-state fermentation, also known by « Koji » process, was first developed in Japan where abundant raw materials such as fruit wastes and mainly rice bran are available. It has been an alternative method for using agro-industrial residues (Xu *et al*., 1989). Solid-state culture is characterized by the development of microorganisms in a low-water activity environment on an insoluble material that acts both as physical support and source of nutrients (Gonzalez *et al*., 1997).

Some similarities between solid and surface process are observed, since the fungus also develops on material surface. The substrate is solid and it is moistened to about 70 % moisture, depending on the substrate absorption capacity. The initial pH of the material is normally adjusted to 4.5–6.0 and the temperature of incubation is about 28–30 °C, depending on the microorganism used (Xu *et al*., 1989). The solid culture process is completed within 96 hours under optimal conditions (Kubicek and Rohr, 1986). The decline in concentration of citric acid higher than the optimum period may have been due to decay in the enzyme systems responsible for the production of citric acid up on exhaustion of fermentable sugars, or could be attributed to citric acid break down due to the toxic effect of accumulated waste product (Roukas,1999; Kristiansen and Sinclair ,1979).

Since it uses agro-industrial residues that are cheaper source of substrates, solid state fermentation process is especially ideal for developing countries like Ethiopia (Amare, 2006).

Ethiopia is the country that imports citric acid from different countries. The amount of citric acid imported from India, Iran, Saudi Arabia and USA to Ethiopia was 6968kilogram in the first quarter of 2010 (table 2). This amount of citric acid imported required about 277525.69 birr expense for buying purpose.

Table 1: Amount of citric acid imported from different countries to Ethiopia

Country	2004		2005		2006		2007		2008		2009	
	Value in birr	Wt(kg)	Value in birr	Wt(kg)	Value in birr	Wt(kg)	Value in birr	Wt(kg)	Value in birr	Wt(kg)	Value in birr	Wt(kg)
United Arab E.	-	-	2415	1323	25001	2500	-	-	2702	75	-	-
Australia	-	-	-	-	31407	20800	-	-	-	-	-	-
China	464	65	37711	3128	153693	14129	285307	33370	570818	27454	824270	81125
German	-	-	-	-	19057	650	27471	806	1454	21	70777	801
Denmark	-	-	7032	145	-	-	-	-	342	7	-	-
Ecuador	-	-	598	10	-	-	-	-	-	-	-	-
Egypt	-	-	350	16	36386	1000	-	-	-	-	-	-
France	-	-	-	-	-	-	45938	338	-	-	-	-
U. K.	2380	80	-	-	-	-	-	-	-	-	177	2
Greece	960	34	-	-	-	-	-	-	-	-	-	-
Israel	2351	100	-	-	4900	300	-	-	-	-	-	-
India	121729	20	1059	125	168	71	29815	699	70106	2455	67078	3065
Italy	-	-	4139	200	-	-	-	-	-	-	5983	125
Kenya	63313	1845	29426	1399	30373	3020	-	-	-	-	-	-
Korea	440266	750	-	-	-	-	-	-	-	-	-	-
Netherland	-	-	-	-	16685	2000	-	-	-	-	-	-
Pakistan	-	-	-	-	-	-	-	-	4067	250	5710	500
Saudi Arab	-	-	-	-	-	-	50338	1298	63711	2857	-	-
S. Korea	256000	13135	-	-	-	-	-	-	-	-	-	-
Singapore	813829	173	-	-	-	-	-	-	-	-	-	-
Turkey	-	-	-	-	-	-	-	-	4783	144	13874	996
USA	-	-	37205	1603	35666	352	167641	2320	111060	3520	98884	1045
S. Africa	-	-	-	-	23035	617	363194	19427	163873	5261	-	-
Total	**1701290**	**16202**	**119936**	**7949**	**376371**	**45439**	**969702**	**58258**	**992917**	**42044**	**1086752**	**87659**

Source: Ethiopian Central Statistics Agency (ECSA), 2010.

Table 2: Amount of citric acid Imported from different countries to Ethiopia in quarter of the year, 2010

Month

Country	January		February		March	
	Value in birr	Wt(kg)	Value in birr	Wt(kg)	Value in birr	Wt(kg)
U. k.	-	-	88.57	1	-	-
India	-	-	-	-	4197.37	188.42
Iran	-	-	-	-	416.79	6
Saudi Arabia	204792.19	6000	-	-	-	-
USA	-	-	-	-	68119.34	774
Total	**204792.19**	**6000**	**88.57**	**1**	**72733.5**	**968.42**

Source: Ethiopian Central Statistics Agency (ECSA) (2010).

Ethiopia produces a large amount of agricultural products and residues. Thousands of tons of agricultural and domestic wastes are produced daily. Thus, there is an urgent need to find suitable applications of these waste products. One alternative for its economic utilization could be to use it as substrate in fermentation processes for the production of value added products like citric acid. Although citric acid production neither from citrus fruits nor by microbial fermentation is started in Ethiopia, following the growth of beverage, food , detergent and other factories, its demand has increased from time to time(table 1and 2). The aim of this study was to produce citric acid in solid state fermentation system using indigenous isolates of fungi, *Aspergillus niger*.

2. OBJECTIVES

2.1 General objectives

The general objective of the research is to produce citric acid in the laboratory from solid substrate by *Aspergillus niger* through solid- state culture method.

2.2 SPECIFIC OBJECTIVES

☞To isolate and characterize *Aspergillus niger* from soil, seed and air samples,

☞To produce citric acid from raw materials such as wheat bran, rice and shredded maize using solid-state fermentation processes in the laboratory,

☞To optimize factors that affect maximum production of citric acid.

3. LITERATURE REVIEW

3.1 History of citric acid

Citric acid fermentation was first observed as a fungal product by Wehmer in 1893 by a culture of *Penicillium glaucum* on sugar medium. After a few years, he isolated two new fungal strains which were designated as *Citromyces* (*Penicillium*) with the ability to accumulate citric acid. However, industrial trials did not succeed due to contamination problems and long duration of fermentation (Mattey, 1992).

It was the work of Currie which opened up the way for successful industrial production of citric acid. In 1916, he found that numerous strains of *Aspergillus niger* produced significant amounts of citric acid. The most important finding was that *A. niger* grew well at pH values around 2.5-3.5 and high concentrations of sugars favour citric acid production. In the 1930s, some units were implanted in England, Soviet Union, and Germany for the commercial production (Mattey, 1992).

Several works were dedicated to the optimization of the conditions for the utilization of cheap materials like sugar cane molasses, beet molasses, starch and hydrolysate starch (Sarangbin *et al*., 1999). Various processes for treating and purifying molasses were developed, especially for the removal of trace metals. Moreover, it was found that a small excess of copper ions was beneficial to achieve high yields of citric acid (Soccol *et al.*, 2006).

Aspergillus niger, the most important fungus used in industrial microbiology, has been employed for many years for the commercial production of citric acid (Schuster *et al*., 2002). It is one of the oldest named genera of fungi, which received its name from Micheli in 1729. In viewing the microscopic spore-bearing structure, Micheli was reminded of a device used by the Roman Catholic clergy to sprinkle holy water during a part of the liturgy called the *asperges* (Ainsworth, 1976).

Citric acid is produced commercially from the fermentation of bulk hydrated materials and by-product of sugar production (Wang, 1998; Lesniak, 2002). Almost entire quantity of citric acid is obtained through submerged fermentation of starch or sucrose based media, using the filamentous fungus *Aspergillus niger* (Vandenberghe *et al.*, 2000b). Solid-state fermentation (SSF) has been an alternative method for citric acid production using agro-industrial residues (Prado *et al*., 2005).

3.2 Extraction of Citric Acid from citrus fruits

Citric acid occurs naturally in various fruits such as lemons, oranges, pears and figs. It was first isolated in the crystalline form by Scheele in 1784 from the juice of lemons. The Scheele process makes use of the insolubility of calcium citrate, which is precipitated from heated lemon juice by calcium hydrate. The boiling is continued to increase the particle size, thus facilitating removal by filtration. Until the 1920s, citric acid was extracted from the lemon juice and crystallized by this process (Papagianni, 2007). Extraction from citrus fruits is possible but it is not cheaper than fungal fermentation. However, a small amount of citric acid, approximately less than 1% of total world production, is still produced from citrus fruits in Mexico and South America where citrus fruits are available economically **(** Yigitoglu, 1992) .

3.3 Microbial production of Citric acid

A large number of microorganisms including fungi and bacteria as shown in table 3 have been employed for citric acid production (Grewal and Kalra, 1995; Yokoya, 1992). Most of them, however, are not able to produce commercially acceptable yields due to the fact that citric acid is a metabolite of energy metabolism and its accumulation rises in appreciable amounts only under conditions of drastic imbalances. Among the mentioned strains, the fungus *A. niger* has remained the organism of choice for commercial production because it produces more citric acid per unit time (Soccol *et al.*, 2006).

Table 3. Citric acid producing microorganisms

Bacteria	Fungi	Yeasts
Arthrobacter	*Aspergillus niger*	*Candida tropicalis*
Parafinens	*A. Acleatus*	*C. oleophila*
Bacillus licheniformis	A. *carbonarius*	*c. guilliermondii*
Corynebacterium sp	*A. Awamori*	*C. citroformans*
	A. Foetidus	*Hansenula anamola*
	A. Fonsecaeus *Penicillium janthinellum*	*Yarrowia lipolytica*

3.4 Yeasts

Alternatively, there is a great interest in the possibility of CA production by different strains of yeast. Yeasts belonging to species *Yarrowia lipolytica*, *Candida guillermondii* and *C. oleophila* are known to be able to produce a wide spectrum of organic acids, including TCA cycle intermediates such as CA and isocitric acid (ICA). The problem in the production of citric acid for yeasts is the simultaneous formation of ICA (Soccol *et al.*, 2006).

3.5 Fungi

Among 200 genera of molds, *Aspergillus* is the most important filamentous fungus. The most common species of this genus is *Aspergillus niger*, which, like other fungi, requires an aerobic environment for growth (Asan, 2004). With carbon rich substrates such as glucose, *A. niger* produces colonies which are powdery in texture, with a black surface color and pale yellow underneath. The morphology of *Aspergillus niger* is complex because it exhibits different

structural forms throughout its growth cycle. The basic differences between *A. niger* and other *Aspergillus* species are colour, growth rate and thermo-tolerance (Papagianni, 2004).

As opposed to *A. niger*, *A. nidulans* produces blue green colonies with a white or brownish underneath. The underneath colour of *Aspergillus* species ranges from uncoloured to pale yellow and even to purple and olive in some strains of *A. nidulans* and orange to purple in *A. versicolor* (Collier *et al*., 1998).

Observed under microscopy, *A. niger* is known to grow relatively rapid with its colony diameter increasing from 1 to 9 cm, over 7 days at 25℃ on Czapek-Dox agar. This can be compared to the growth rate of *A. nidulans* and *A. glaucus* which can increase their colony diameter from 0.5 to 1 cm under the same incubation conditions (Larone, 1995). *A. niger* is considered a mesophilic fungus, and can be distinguished from others on the basis of temperature tolerance range. On agar, *A. niger* tolerates temperatures in the range of 25 to 35℃, with 30℃ being ideal (Hayden and Maude, 1994). Other fungi, such as *A. fumigatus*, grow well at temperatures over 40℃ (St-Germain and Summerbell, 1996).

In submerged conditions and during fermentation, the morphology of *A. niger* can range from pellets to free filaments. Free filaments in the form of mycelia or single hyphae with conidiophores occur when the nutrient solution is mixed at a high speed, shearing the hyphae from one another while pellets are formed under mild mixing conditions (Pazouki and Panda, 2002). For citric acid production under submerged conditions, the pellet form is preferred. When the nutrient solution is not mixed, the fungi tend to grow at the liquid surface forming a mat of mycelium (Steel *et al*., 1954).

In solid state fermentation, substrate availability for enzymatic hydrolysis is important. One of the main problems in SSF with filamentous fungus, both in laboratory and industrial scale is the estimation of biomass, as the methods used in liquid fermentation cannot be applied. This fact is

due to the intense adhesion of filamentous fungus mycelium to the solid substrate/support and to the SSF system heterogeneity (Vandenberghe *et al*., 2000). Oxygen consumption and CO_2 production is a result of metabolic activity of microorganisms from which they obtain the necessary energy for growth and maintenance. Besides, the metabolic activity is associated with growth and it can be employed for biomass biosynthesis estimation (Raimbault *et al*., 1997). Several authors such as Pintado *et al*. (1998) used respirometry to follow the gas effluents (CO_2 and O_2) from the bioreactor in order to control fermentation and to evaluate different microbial activities (Soccol, 1992).

3.6 Growth Stages of *Aspergillus niger*

Fungi generally exhibit four basic growth stages: the spore which is a dormant phase, followed by the spore germination or lag phase, the growth or hyphae phase, and the spore formation phase. During the growth phase, the fungus develops tubular filaments known as hyphae, and these hyphae branch out repeatedly to form a mass know as the mycelium. During this phase, the rate of production of biomass is exponential. Citric acid production is known to occur when fungal growth is limited (Vaija and Linko, 1986).

New organisms originate from spores produced asexually by a mature *A. niger* mycelium. To reproduce, fungi develop special hyphae called conidiophore which stand erect and develop numerous spores at their tip. These spores are released into the environment and will remain dormant until proper conditions (moisture, light, temperature, and substrates) allow for their development (Nason, 1968).

Spore germination is generally initiated by a swelling and the division of the nucleus, the production of endoplasmic reticulum, ribosomes and mitochondria. The introduction of any microorganisms, including *A. niger*, to a new environment requires the synthesis of enzymes and intermediates to initiate nutrient ingestion for growth. The duration of the lag phase therefore depends on several factors, including environmental conditions and level of inoculums (Papagianni, 2004; Hossain *et al*., 1984).

The lag phase is followed by the exponential growth of the fungus *A. niger*, when growth conditions are favorable and nutrients are available. During exponential growth, hyphae grow in length at their tips, branch out and extend outwards. The fungal hyphae responsible for the supply of nutrients will penetrate rich solid substances (Jackson and Heath, 1993).

The exponential growth of the fungal mass may not be uniform within a colony. The older and central hyphae may exhaust their nutrient supply while the hyphae found at the surface may access new sources of nutrients and maintain their growth (Moore-Landecker, 1996). When nutrients become limited, especially for the older hyphae, a stationary phase is exhibited where growth is virtually stopped. This phase establishes the balance between hyphal mass increase and decrease.

3.7 Biochemistry of Citric Acid Production by *Aspergillus niger*

Citric acid or citrate, the ionic form of citric acid, is a major intermediate compound produced by the tri-carboxylic acid (TCA) cycle. Pyruvate ($HO(CO)_2\ CH_3$), a product resulting from the oxidation of glucose, namely glycolysis is the compound required to initiate the TCA cycle. Pyruvate must first be converted to acetaldehyde (CH_3COH) and then Acetyl CoA (figure 1) to activate the TCA. The first intermediate compound produced by the TCA cycle is citrate ($C_3H_5O(COO)_3^{-3}$) which is the ionic form of citric acid ($CH_2HOC(COOH)_3CH_2$). In *A. niger*, the TCA cycle occurs in the matrix of the mitochondrion (Dunn, 1998).

Once formed, Citrate can be used as a major intermediate in the TCA cycle, to generate oxaloacetate and several compounds used to store energy such as ATP and NADPH or can be excreted outside the cell wall, if conditions prohibit the second stage of the TCA cycle. If nutrients become limited, the citrate accumulated outside the cell wall can be reabsorbed and degraded within the TCA cycle by the enzyme aconitase (Dunn, 1998).

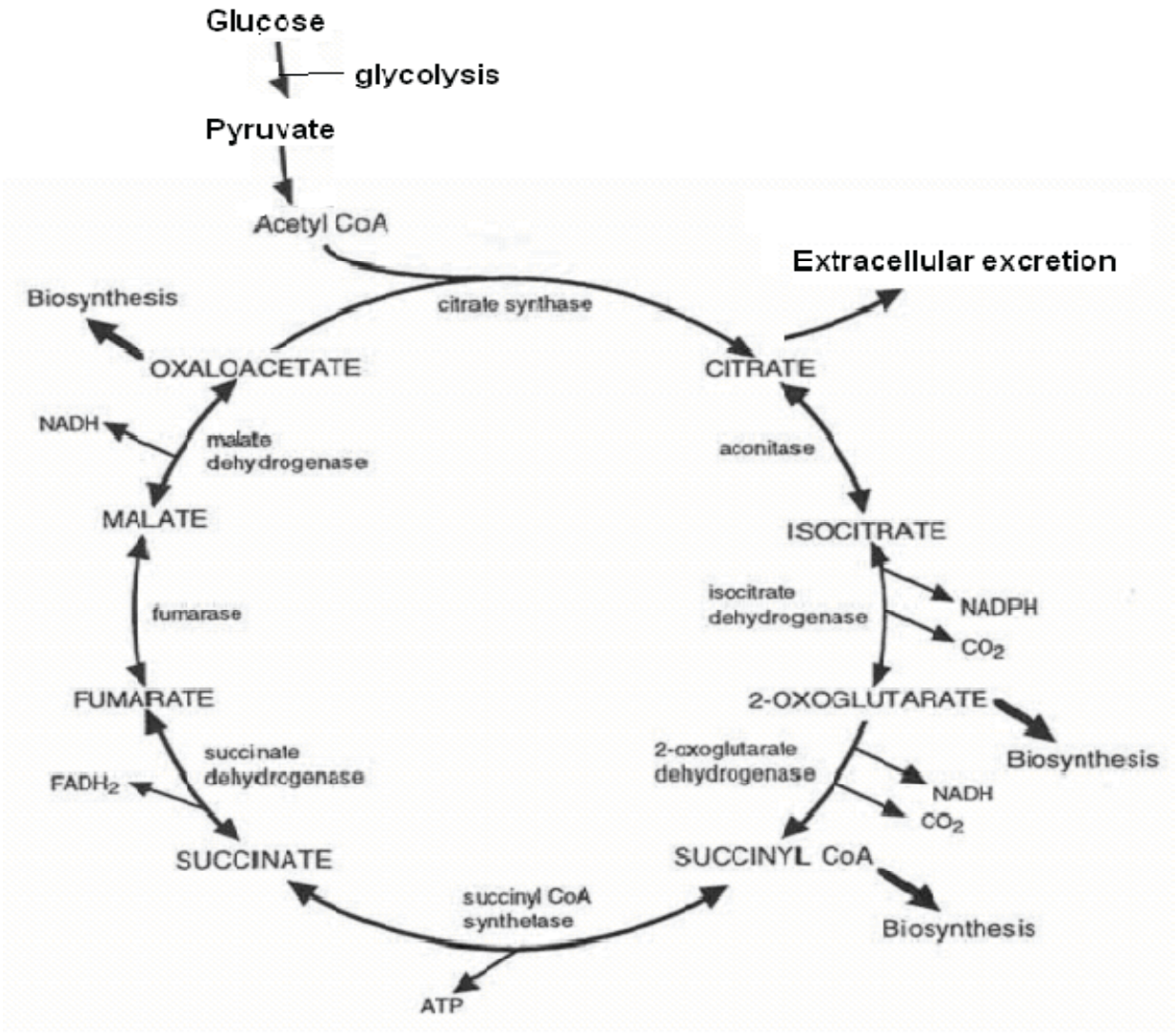


Figure1: The Tri-carboxylic acid (TCA) cycle, responsible for the production of citrate.

3.8 Solid state fermentation

Although most of the biotechnology industries relied on submerged liquid fermentation process (SLF), some of the biotransformation reactions can be carried out in a different fermentation process that is known as solid-state fermentation (SSF). SSF proves to be a better option than the SLF (table 4). Interest in SSF has been increasing because of its important applications in producing enzymes, bio pesticides, aroma compounds, biopharmaceuticals, organic acids and other bioactive compounds. Agro-industrial residues are processed using SSF because it has

lower energy requirement, produce lesser waste water and are environment-friendly (Manpreet *et al*., 2005).

The major difference between SSF and SLF is the free water content in the substrate. The term solid-substrate fermentation is used in a more general sense to describe any process in which solid particles of substrates were involved, regardless of the amount of free water. Therefore, SSF includes the processes in a solid matrix with an aqueous phase leaching through it, slurries of solid particles and solids suspended in an aqueous phase (Moo -Young *et al*. 1983).

In the case of SLF, substrate is freely accessible to the microorganisms while in SSF the availability of substrate to the microorganisms may increase, decrease, or remain relatively constant during the fermentation (Knapp and Howell, 1985).

SSF stimulates the growth of microorganisms in nature on the moist solids and has been credited to be responsible for the beginning of fermentation technique in ancient time (Cannel and Moo-Young, 1980).The first milestone in the development of SSF was achieved during 1950-1960, when steroid transformation was reported using fungal cultures. The trend continued with the production of mycotoxins and protein rich feed, which involved utilization of agro-industrial residues (Narayanamurthy *et al*., 2008). This was the time when SSF was actively researched, followed by numerous patents and publications on basic aspects of SSF (Han *et al.*, 2001).

Several authors had reviewed the history and development of SSF. Aidoo *et al*., (1982) made an attempt to trace the history of growth of microorganisms on solid substrates and the term solid-state fermentation came after this. Cannel and Moo-Young in 1980 reviewed the developments of SSF in food industry and composting. They compared SSF with SLF and also described the advantages and disadvantages (table 4).

Table 4. Differences between Solid-state and Submerged liquid Fermentation

Solid-state fermentation(SSF)	Submerged liquid fermentation(SLF)
• Organisms requiring less water for growth are preferred such as filamentousfungi. •Inert support (natural or artificial), containing all components for growth in the form of solution. •Less chance of contamination because of low availability of water. • Small sized bioreactors can be used. • Less consumption of energy for aeration and gas transfer. • Limiting factor for growth is diffusion of nutrients. • Lots of difficulties in measuring the quantity of biomass present and other online processes. • Downstream processing is easy, cheaper and less time consuming. •Liquid waste is not produced	• Media concentration is very much lower as compared to water content. •Required processed ingredients are expensive. • Higher water activity becomes the major cause of contamination in SLF. • Large-scale bioreactors are required because media is very much diluted. • High air pressure consumes more power and there is power transfer of gas in SLF. • Vigorous mixing makes diffusion easy. • Online sensors are available and sampling is easy for biomass measurement. • Water makes downstream process difficult and very expensive. • High quantity of liquid waste is produced and causes difficulties in dumping.

3.9 An overview of SSF process

The important aspects which should be considered before starting a solid-state fermentation include the selection of suitable microorganism and substrate, optimization of process parameters and isolation and purification of the product (Manpreet *et al*., 2005).

SSF follows the same steps as in the SLF, namely:

•Substrate preparation,

•Inoculums preparation,

• Cultivation within a bioreactor,

•Downstream processing and,

•Product recovery.

Fungi and yeast seem to be favorable than bacteria because of their ability to grow on the substrates, which are low in water content. Due to high water activity requirement, bacterial cultures might not be suitable for SSF. However, Pandey in 1992 reported that bacterial culture can be well managed and manipulated for SSF process.

Another aspect that affects the production is the selection of proper substrate. Substrate in SSF is non soluble material that acts both as physical support and source of nutrients. Byproducts of agricultural activities or the products obtained after the processing of agricultural materials can be used as substrates. Some of the substrates may require pretreatment. Chopping or grinding may be necessary to reduce particle size, cracking may be necessary to increase the accessibility of the interior of the particle to the microorganism, thermal pretreatment or even chemical hydrolysis might be necessary to increase the susceptibility of macromolecules within the solid to attack by microbial enzymes (Mitchell *et al.,* 2000).

The second thing one has to consider is the production of a specific product from a suitable substrate. For this reason, different substrates have to be screened for a specific product. Similarly, it would be important to screen different microorganisms and select the most suitable one. Product isolation could be relatively tougher and more costly when using naturally occurring raw materials such as wheat bran as the substrate, because while extracting the product after fermentation, along with the product, several other water-soluble components from the substrate also leach out and may pose difficulties in the purification process (Moo-Young *et al.,* 1983).

Selection of process parameters and their optimization are other key aspects of SSF. This part includes physiochemical and biochemical parameters such as particle size, initial moisture content, initial pH, relative humidity, incubation temperature, agitation, aeration, age and size of inoculums, supplementation of nutrients and trace elements, supplementation of additional carbon source and inducers, extraction of product and its purification (Manpreet *et al*., 2005).

3.10 Microorganisms suitable for SSF

The low amount of free liquid in the substrate affects the whole process of SSF and therefore it becomes the most important feature of SSF. All the factors in SSF depend up on the amount of water present in the substrate. Care should be taken while selecting the microorganism for fermentation on solid-substrates (Doelle *et al.*, 1992).

Due to the less availability of free water in SSF process than the majority of liquid fermentations, most of the SSF processes involve fungi, although there are number of reports involving bacteria and yeast. Bacteria are mainly involved in processes like composting; ensiling and yeasts have been used for ethanol and food or feed production (Doelle *et al.*, 1992).

The yeast grows on solid-substrate as minor member and the dominant species are bacteria for example *Lactobacilli* in ensiling and in tape manufacturing (Moo-Young *et al*., 1983). *Saccharomyces cerevisiae* has been employed in semi-solid fermentation of grape pomace and for SSF of sweet sorghum, sugar beets (Kirby and Mardon, 1980) and fodder beets (Gibbons *et al.*, 1984).

A number of bacterial species have been employed for solid-substrate fermentation using many substrates. *Lactobacilli* are used for ensilage and thermophilic bacteria predominate in composting. *Bacillus subtilis* is the major organism used for production of Japanese food *Natto* (Beuchat *et al*., 1985). *Lactobacillus plantarum* and *Propionibacterium shermanii* are used to ferment and preserve high moisture grain (Flores *et al.*, 1985).

Out of all the groups of fungi, the filamentous fungi are the major group of microorganisms, which predominate in the SSF process. Different species of fungi used in SSF process include many species of *Aspergillus, Rhizopus, Alternaria, Fusarium, Monilia, Mucor, Trichoderma* and some species of *Penicillium*(Manpreet *et al*., 2005).

Most of the species belong to filamentous fungi, as these are best suited because of their ability to spread over and to penetrate inside the solid-substrate. The other advantage of using

filamentous fungi is that the fungal mycelia synthesize and release large quantity of extra-cellular hydrolytic enzymes (Lockington *et al*., 2000).

3.11 Substrates used in solid state fermentation

Substrates used in SSF are generally insoluble in water. In practice, water is absorbed onto the substrate particles, which can then be used by microorganisms for growth and metabolic activities. Bacteria and yeast grow on the surface of the substrate while fungal mycelium penetrates into the particles of the substrates (Pandey, 1992).

Substrates for SSF can be divided into three groups, starchy substrates, cellulose or lignocellulose and those with soluble sugar (table 5).

Table 5. Examples of different types of substrates for SSF

Starchy	Cellulose/ lignocelluloses	Soluble sugar
Rice	wheat straw	Grape pomace
Cassava	Corn	Sweet sorghum
Wheat bran	Rice stover	sugar-beet
Rice bran	wheat bran	Pineapple waste
Buckwheat seeds	sugar-beet pulp	Carob pods
Corn meal	Wood	Coffee pulp
Sweet potato residue		Molasses
Banana meal		

Source: Weber *et al*., 1999

Another strategy for SSF is to impregnate inert solid material such as bagasse or hemp with soluble sugars in order to provide a SSF environment for the growth. Natural substrates are easily available and are cheaper than synthetic substrates. But, they generally require pretreatment to make their chemical constituents more accessible and their physical structure more susceptible to mycelial penetration (Weber *et al.,* 1999).

Physical treatment includes chopping or grinding to reduce size and cracking to make the interior of the particle more accessible. Chemical treatment includes high temperature cooking and acid or alkali treatment. Supplementation of additional nutrients may be required in order to stimulate growth, induce enzyme synthesis or to prolong secondary metabolite production such as the supplementation of 0.5% of glucose or cellobiose, 0.5% peptone, asparagines or yeast extract are in use (Georgiou and Shuler, 1986).

Physical factors affecting utilization of solid-substrate include accessibility of substrate to the microbes (porosity and particle sizes affect the accessible surface area to the organisms), film effect and mass effect (Knapp and Howell, 1985).

The size of substrate particles affects the extent and rate of microbial colonization, air penetration, CO_2 removal and downstream extraction. The optimum particle size often represents a compromise between the accessibility of nutrients and the availability of O_2. Particle size from 1 mm to1cm is often used in SSF. Knapp and Howell (1985) reviewed the effect of alteration of substrate particle size on SSF. Pandey (1992) has reported that productivities were higher with the substrate that contains particles of mixed sizes varying from 180 µm to 1.4 mm.

Chemical factors affecting utilization of solid-substrate include degree of polymerization and crystallinity. The selection of substrates and optimization of its concentration plays an important role in yielding the higher growth rates of microbes. At higher growth rate, the ratio between heat losses to heat production is low and therefore helps to approach the behavior of large scale bioreactor (Manpreet *et al*., 2005).

3.12 Temperature

Microbial growth in SSF generates significant amount of metabolic heat. It has been reported that 100-300 kJ of heat per kg of cell mass is generated in a SSF process. Establishment of temperature gradients and localized overheating of the substrate occurs because of inefficient removal of heat from the substrate (Prior *et al.*, 1992).

Heat transfer problem in SSF includes temperature gradients that may cause belated microbial activity (Ikram-ul-Haq *et al*., 2002), dehydration of the medium and undesirable metabolic deviations (Rathbun and Shuler, 1983). Heterogeneous materials make heat removal difficult; this is due to low transfer coefficient and low thermal conductivity (Narahara, 1984).

Temperature can rise rapidly, because there is little water to absorb the heat or in other words mean specific heat capacity of the fermenting mass is much lower than that of water. Therefore, heat generated must be dissipated immediately as most of the microorganisms used in SSF are mesophilic, having optimal temperature for growth between 20 and 40ºC and maximum growth below 50ºC(Saucedo-Castaneda *et al.*, 1990) .

Different methods are used for dissipation of heat such as cooling of metal trays using circulating coolant and intermittent agitation. Other methods include forced aeration, water circulation through a jacket surrounding the fermenter, agitation of the solids and covering the external surface of fermenter with water soaked burlaps. However, forced aeration is generally the technique of choice for heat removal (Barstow *et al.*, 1988).

3.13 Effect of pH on SSF

There are remarkable changes, which occur in the pH of the substrates. These are mainly for the production of acids due to incomplete oxidation of the substrate or uptake of ammonium ions, which will cause the pH to fall, while the release of ammonia by deamination of urea, or other amines will increase the pH (Raimbault and Alazard, 1980).

As we cannot monitor pH in the SSF it is very difficult to control it. So, it is desirable to use microorganisms which can grow over a wide range of pH and which have broad pH optima. By using different ratios of ammonium salts and urea in the substrate, pH control in both natural and model SSF system can be obtained (Raimbault and Alazard, 1980).

The effect of increasing buffer concentration, effect of pH on the maximum specific growth rate and the optimization of different buffers for the growth and enzyme parameter were investigated by Nagel *et al.,* (1999). They have also calculated the required buffer concentration by utilizing expected biomass production, initial pH and pH change.

Exact control of pH is very difficult in SSF process, but one can maintain pH during the process by using pH-correcting solutions. Many substrates are effective buffers. This is particularly true for protein rich substrate, especially if deamination of protein is minimal (Mitchell *et al*., 1991).

3.14 Moisture

Water activity (aw) of the moist solid substrate is the ratio of vapour pressure of water above the substrate in a closed system to the vapour pressure of the pure water at same temperature. It is measured as relative humidity divided by 100. Pure water has a_w = 1.00 and a_w decrease with the addition of solutes. Bacteria mainly grow at higher a_w values, while filamentous fungi and some yeast can grow at lower a_w value (0.6-0.7). The microorganisms, which can grow and are capable of carrying out their metabolic activities at lower a_w values, are good for SSF processes (Prior *et al.*, 1992; Nampoothiri *et al*., 2003; Battaglin *et al*., 1991).

For fungi, the optimal moisture requirement varies between 40 and 80% (w/w), but for the same organism growing on different substrate, the optimum moisture level may differ widely, hence, the amount of moisture by itself is unreliable for predicting growth (Prior *et al.*, 1992).

It has been shown that in the course of fungal growth in SSF, higher water activities favour sporulation while low water activities favour spore generation or mycelia growth (Gervais *et al*., 1988; Parra *et al*., 2004).

3.15 Applications of SSF

SSF was a traditional process, but the exact potential of SSF was only known during 21st century. The traditional Koji process may be considered the archetype of SSF started in 7th

century in Japan. Its importance is because of the high content of amylolytic and proteolytic enzymes, which catalyze the degradation of starches and proteins (Manpreet *et al*., 2005).

Koji production is done using strains of *Aspergillus oryzae* on steamed rice and incubated under controlled temperature and humidity. A Japanese biochemist and industrialist leader Dr. Jokichi Takamine began commercial production of koji from the fungus *Aspergillus oryzae* and called it "takadiastase" introduced koji in USA in the year 1891 using wheat bran. Most of the cheese such as blue-veined cheese using *Penicillium roqueforti*, Camembert and Brie cheese using *Penicillium camemberti* and *Penicillium caseicolum* are produced by SSF (Mial, 1975).

SSF is also widely employed in processes like ensiling, composting using thermophilic bacteria, mesophiles, actinomycetes and fungi. Nowadays, lots of byproducts such as enzymes, organic acids, ethanol, biogas, antibiotics, surfactants, toxins, bioremediation agents, mushrooms, compost, microbial polysaccharides, biopesticides, protein-enriched fermented foods, predigested feeds for ruminants are produced by SSF process (Stentiford and Dodds, 1992).

In recent years, considerable interest has been shown in solid state production of citric acid by *Aspergillus niger* using agro residues like bagasse, corncob, carob pod (Roukas, 1999; Vandenrberghe *et al*., 2000) and waste of food processing industries like apple and grape pomace and fruit peel (Shojaosadati and Babaeipour, 2002) due to its several advantages like solid waste management, biomass energy conservation, production of high value products and little risk of bacterial contamination (Roukas, 1999; Tran and Mitchell, 1995).

4. MATERIALS AND METHODS

4.1 Sample collection

All samples (seed, soil and the environment) were collected from Addis Ababa, Yeka and Arada sub Cities, in November, 2009. Seed samples were collected from a milling house, located around Arat Kilo; Soil samples were collected from one inch below the soil surface in sterile bags from Addis Ababa University Science Faculty green house and from farm lands in Yeka sub city the so called Gurara, specific area, Ankurcha. All of them were taken to the Mycology Laboratory, Addis Ababa University, Science faculty and stored in refrigerator at 4ºc for further studies. Air samples were collected by placing the Petridish containing Potato Dextrose Agar media (PDA) in the air for about 1hr.

4.2 Media preparation

To isolate *Aspergillus niger* strain from the collected soil, seed and the environment, Potato Dextrose Agar (PDA) and Czapek Dox Agar (CZA) were used. Potato Dextrose Agar (PDA) was composed of: Potato, 100g; Dextrose, 10g; Agar, 10g and distilled water, 500ml and CZA was composed of : sucrose, 15g; $NaNO_3$, 1.5g; KH_2PO_4, 0.5g ; $MgSO_4$, 0.25g; KCl, 0.25g; Agar, 7.5g; Ferrous Sulfate, 7.5mg and distilled water, 500ml.

4.3 Isolation of *Aspergillus* niger strains

Aspergillus niger strains from soil samples were isolated using soil dilution plate technique. 10g of each soil sample was suspended in 90ml of sterile distilled water, shaken on an orbital shaker GALLENKAMP (London) at 121rpm for about 1hr. One ml of the sample was taken and serially diluted in to 6 test tubes each containing 9ml of sterile distilled water. O.1ml of the sample was taken from 10^{-4}, 10^{-5}, 10^{-6} dilutions and was spread plated on each plate containing potato dextrose agar medium. The plates were incubated at 30°c and examined daily for 7days. Isolates from seed samples were isolated by inoculating the washed seeds in 70% ethanol and distilled

water in to PDA and incubating for 7days similarly as in the soil samples. The inoculated PDA slants were incubated for 7 days and then stored at 4 $^{\circ}$c for further use.

4.4 Slide culture preparation

In a small Petridish, after placing a sterile filter paper and some sterile distilled water, a few centimeters apart, two pieces of glass rods were placed on the filter paper. Clean, alcohol flamed and heat sterilized glass slide was placed on the rods. Using a sterile spatula, a small square (about 1 x 1 cm) of agar block was cut from the Potato Dextrose Agar (PDA) and placed on the centre of the slide. Using a needle, the agar was inoculated with a small amount of *A. niger* strain under test on each of the four sides of the block. A heat-sterilized cover slip was placed over the block and pressed down gently. It was incubated at 28^{o}C and examined daily for sufficient growth (Figure 2). When good growth was appeared, a drop of Lacto Phenol Aniline Blue (LPAB) was placed on a clean slide, the cover slip was removed using forceps and passed the top side of the cover slip in front of the incinerator opening to fix and placed it over the LPAB on the slide. The preparation was examined under the light microscope.

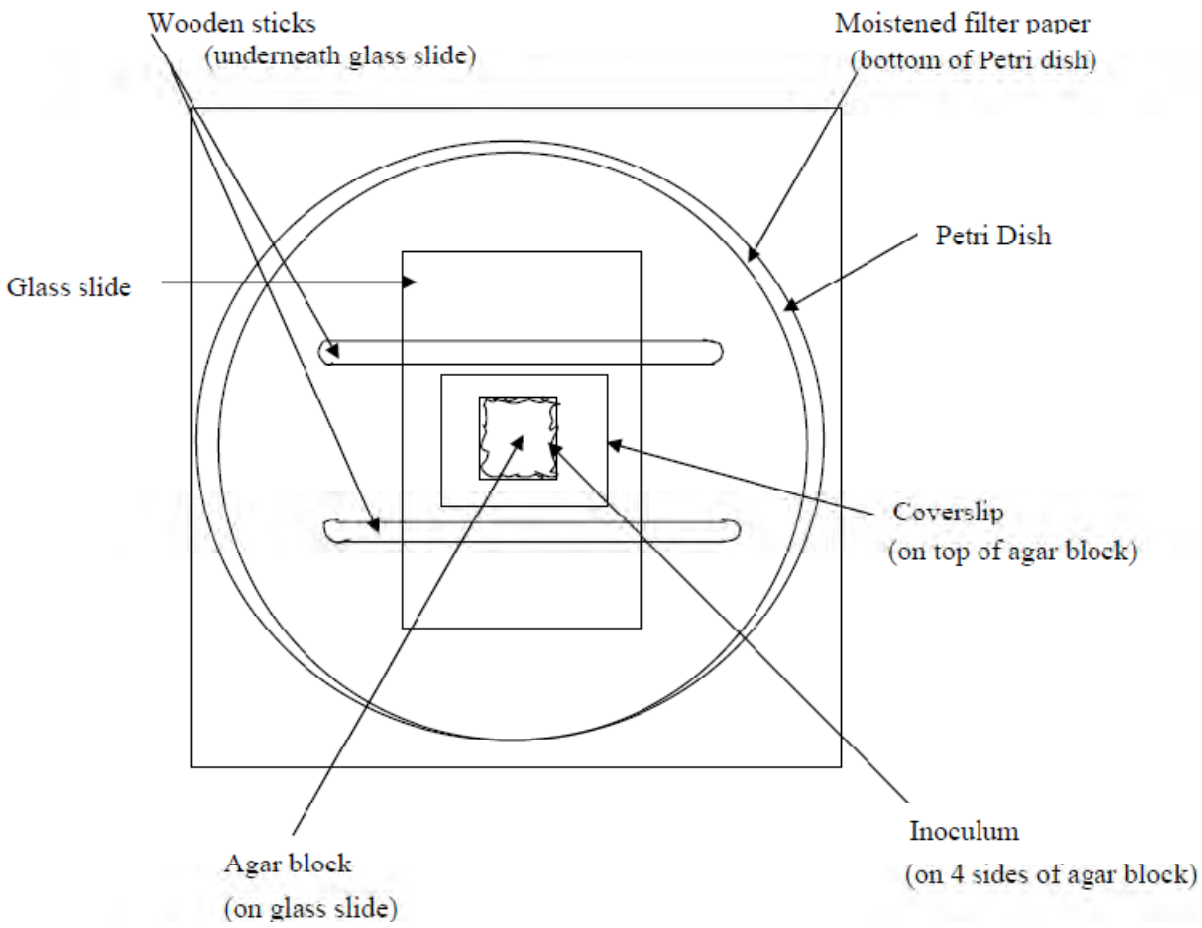


Figure 2: Setting up the slide culture

All isolates, *A.niger* isolate from air sample (ASAS), isolate from barely seed(ASBS), isolate from coffee seed(ASCS), isolate from green house soil(ASGS) and isolate from maize growing soil(ASMS) were identified as *Aspergillus niger* strains based on their colony morphology, colour of hyphae, and colour of aerial and substrate mycelium (Williams and Cross, 1971).

4.5 Preparation of spore suspension

Spores were harvested by flooding the plates with sterile distilled water containing 0.05% Tween 80 as a wetting agent, after which spores were scraped from the surface of the colonies with a sterile spatula. The resulting suspension was shaken in a 100ml Erlenmeyer flask to break up the spore chains. The concentrations of spores were determined using a haemocytometer, after which

the suspension was further diluted in sterile Tween 80 solution to achieve the desired concentration (10^7spore per ml).

4.6 Substrates for citric acid production

Shredded maize (M), wheat bran (W), Rice(R) and their different combinations were tried for citric acid fermentation. The experiment was conducted in 250ml flasks containing 10gms of the substrates. Each 10gms of MWR (1:1:1), MW (1:1:0), MR (1:0:1), WR (0:1:1), M (1:0:0), W (0:1:0) and R (0:0:1) were compared with each other to get effective substrate for citric acid production.

After drying at 105ºc to constant weight, the substrates were moistened with 40ml salt solutions containing (g/l) :($NH4)_2SO_4$, 5; KH_2PO_4, 5; MgSO4 .$7H_2O$, 1; NaCl, 1; $FeSO_4.7H_2O$, 1 and sucrose, 100g/kg. Methanol 3% (v/w) was added to each substrate before inoculation. The initial pH of the substrates was adjusted to about 4.5 with NaOH or HCl. Before inoculating with the isolates, the substrates were autoclaved twice at 121ºc for 20 minutes. The flasks were cooled to ambient temperature and inoculated with 1ml of spore suspension containing about 10^7 spores per ml and incubated at 30ºC in an incubator for 3 days by agitating the flasks at 12hrs intervals (Dhandayuthapani, 2009).

Figure 3: Maize, Wheat bran and Rice substrates used for citric acid fermentation

4.7 Citric acid production method

Solid state fermentation method was used for the production of citric acid in the laboratory using 250 Erlenmeyer flask as the small scale laboratory fermenter. The substrates were moistened to appropriate moisture content (Dhandayuthapani, 2009).

4.8 Citric acid production in solid state fermentation

To 10g of substrate in Erlenmeyer flask (250 ml), 40ml stock mineral salt solutions were added to give a final salt concentration of (g/l): ($NH4)_2SO_4$, 5; KH_2PO_4, 5; MgSO4 .$7H_2O$, 1; NaCl, 1; $FeSO_4.7H_2O$, 1 and sucrose 100g/kg (Dhandayuthapani, 2009). After sterilization by double autoclaving, the flasks were cooled and inoculated with 2 ml inoculums culture and incubated at 30ºC for 72 hr. At the end of the fermentation, 100ml of distilled water was added to the flask and shaken for 60 min to extract the impregnated citric acid to the mycelia. The broth was filtered with mash followed by what man no 1 filter paper and the supernatant was analyzed for citric acid content (Vandenberghe *et al*., 2000).

4.9 Effect of moisture level on production of citric acid

The effect of moisture level on citric acid production was tested by varying the substrate to moisture ratio in the range of 1:1 to 1:9 (w/w). Moisture levels 50, 60, 70, 80 and 90% as well as temperature 30ºc was used for moisture content study. All the liquid added into the flask was taken in to consideration in calculating the substrate to moisture ratio (Amare, 2006).

4.10 Effect of fermentation period on production of citric acid

A 10g sample of substrate was mixed with 40 ml of mineral salt solution in 250 ml flask, autoclaved twice, inoculated with 2 ml of culture and incubated at 30ºC. Depending on the pH range, HCl/NaOH was added to the medium after the first autoclaving and cooling, to adjust the

pH to about 5. The contents of the flasks were harvested and assayed at 24 hr interval (Amare, 2006).

4.11 Effect of Incubation temperature on production of citric acid

The influence of temperature on the citric acid production by *A. niger* isolate (ASGS) was studied by incubating at different temperature(25ºc, 30ºc and 35ºc) for 72 hr. Depending on the pH range, HCl/NaOH was added to the medium after the first autoclaving and cooling, to adjust the pH to about 4.5 (Narayanamurthy *et al*., 2007).

4.12 Effect of pH on production of citric acid

The effect of pH on the production of citric acid was determined at various pHs using NaOH and HCl. pH adjustment was done after the first autoclaving of the substrate. To increase the pH, 1N NaOH was added and to decrease the pH, 1N HCl was added drop by drop using a dropper. The pH electrode was carefully inserted in to the Erlenmeyer flask containing the solid substrate, and then slowly moving the flask in order to cover the electrode with the substrate. After the electrode was covered with the substrate, the pH reading was measured repeatedly (Lonsane *et al*., 1992). The pH was measured by using a Jenway 370 pH meter.

4.13 Citric acid standard preparation

Citric acid standard was prepared from citric acid monohydrate (E. Merck, D-6100 Darmstadt, F.R. Germany) by dehydrating it for three days at 90ºc to get anhydrous citric acid. Anhydrous citric acid obtained by heating the monohydrate at 90ºc to constant weight about 72hr was used to prepare a stock solution (50mg/lm) which is stable for at least one year at 0ºc . Standards prepared from the stock solution are stable for one month at 0ºc. Using dilution rule, $M_1V_1=M_2V_2$. To make 0.3mg/lm, 0.25mg/lm, 0.2gm/lm, 0.15gm/lm, 0.1mg/lm and 0.05gm/lm citrate solution, 60µl,50µl, 40µl,30µl,20µl and 10µl per 10ml distilled water was used respectively. The standard citrate solutions were determined photometrically using pyridine and acetic anhydride (Marrier and Boulet, 1958) (Figure 4).

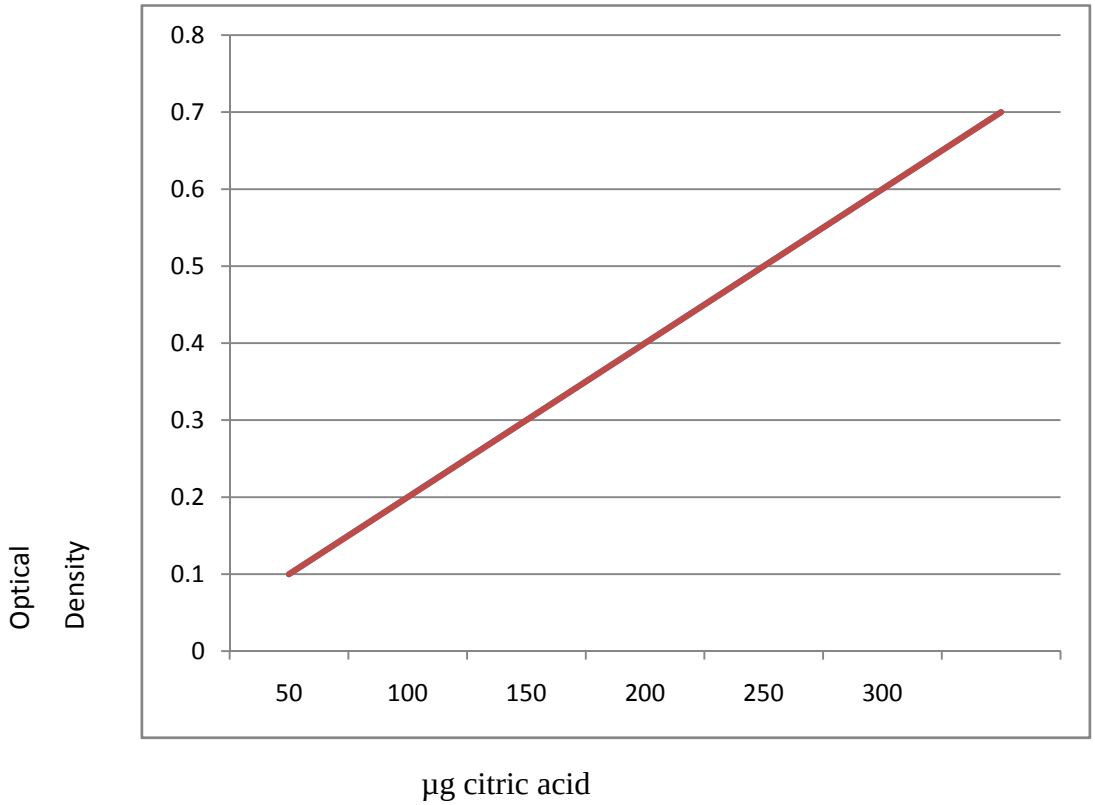


Figure 4. Citric acid standard curve

4.14 Analytical techniques

At the end of the incubation period (optimum 3 days), the fermentation flask was taken out of the incubator. To 10g of fermented product in the flask, 100ml of distilled water was added. After loosening the impregnated mycelium with the substrate using a glass rod, the flask containing the broth was shaken on an orbital shaker GALLENKAMP (London) at 121rpm for 60min. The broth was filtered with mash followed by what man number one filter paper. The supernatant was tested for the pH and stored at 4°C for citric acid analysis (Xu *et al*., 1989).

Citric acid content was determined by spectrophotometer at 420 mµ using Jenway 6405 UV/Vis spectrophotometer after adding pyridine and acetic anhydride. For each 1 ml of sample, 1.3 ml of pyridine (Sigma, St. Louis, MO, USA) and 5.7 ml of acetic anhydride (RESEARCH-LAB FINE

CHEM INDUSTRIES Mumbai 400 002, INDIA) were added to develop color (Marrier and Boulet, 1958).

4.15 Recovery of citric acid

Citric acid recovery trial was done using precipitation (lime-H_2SO_4) method. After completion of step 4.14, the supernatant was treated with $Ca(OH)_2$ and H_2SO_4 . On addition of calcium hydroxide slowly, by stirring using a magnetic stirrer, the filtrate was heated at 50ºc for 20min. Following the addition of $Ca(OH)_2$, sulfuric acid was added drop by drop and shaken by a magnetic stirrer while the flask was in the oven at 65ºc for 60min. Measured supernatant of the fermented broth was precipitated by dosing $Ca(OH)_2$ at 50^oC for 20 min. The precipitated calcium citrate was filtered, dried and weighed. This filtrate was then dissolved in minimum distilled water and precipitated as $CaSO_4$ by adding concentrate H_2SO_4 and shaking the flask with magnetic shaker at about 65^oC for 60min. CaSO4 was filtered and filtrate containing citric acid was further crystallized, re-crystallized, dried and weighed (Soccol *et al.*, 2006).

5. RESULT

5.1 Isolation of *Aspergillus niger*

A total of five *A. niger* isolates were isolated from seed, soil and environment (Figure 5). Two isolates, ASMS and ASGS, were collected from Ankurcha soil and garden soil of the Faculty of Science respectively. Two isolates, ASBS and ASCS, were isolated from barely and coffee seeds respectively. One isolate, ASAS, was recovered from the Mycology laboratory of Addis Ababa University.

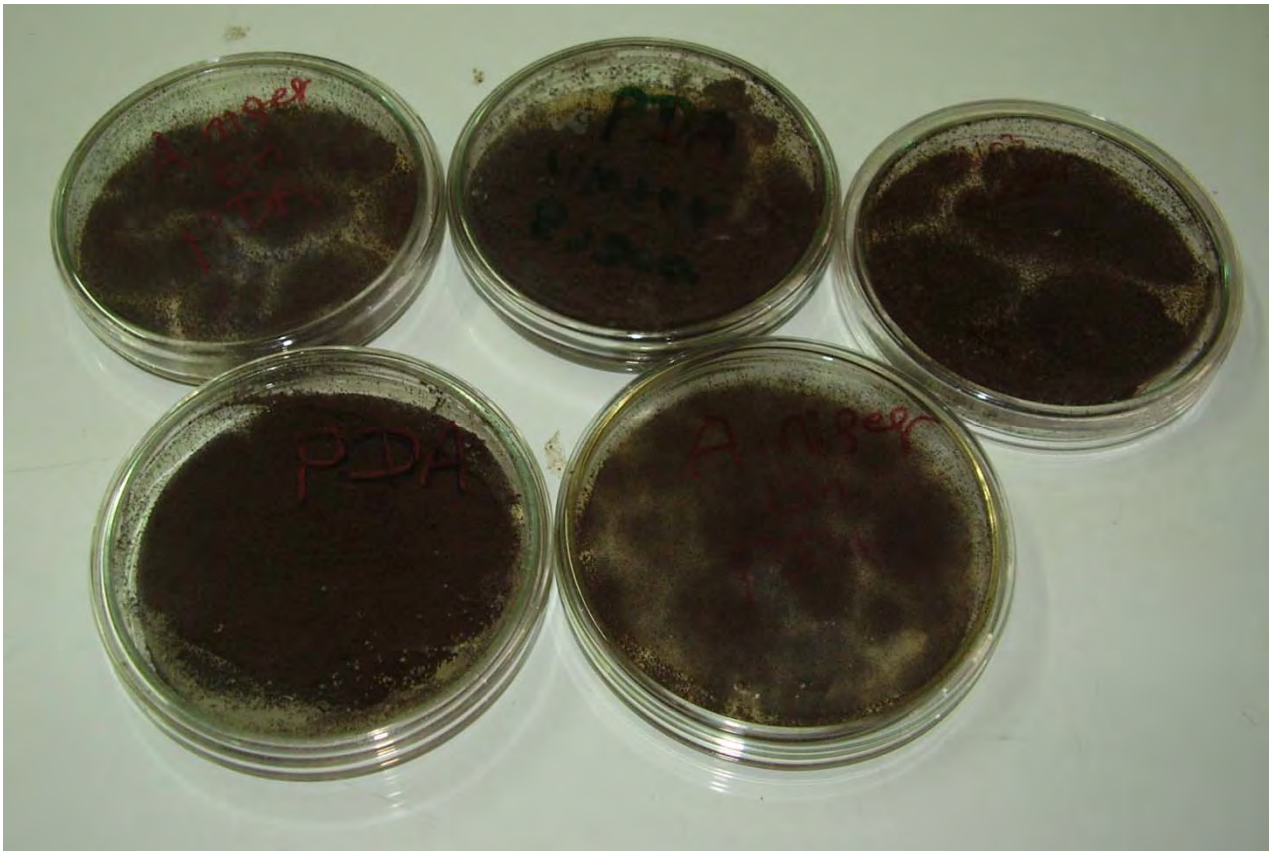


Figure 5: *Aspergillus niger* strains on potato dextrose agar medium

5.2 Screening of *Aspergillus niger* isolates for citric acid production

All the five isolates were screened for citric acid production on rice solid substrate. All of them produced citric acid as shown in (table 6). Two strains, ASCS and ASGS, produced appreciable

amount of citrate on rice substrate. Though, all the isolated strains could produce citric acid, one isolate, coded as ASGS was selected for further study based on its high potential for citrate secretion.

Table 6. Optical density, pH at the end of fermentation and Citric acid (g/kg DR)

Strain	O.D.	pH at the end of fermentation	Citric acid (g/kg DR)
ASAS	0.05	2.36	16.8
ASBS	0.12	2.23	40.2
ASCS	0.16	2.39	53.6
ASGS	0.33	2.15	110.6
ASMS	0.06	2.31	20.1

5. 3 Taxonomic identification of the most effective citric acid producer isolate, ASGS on rice

Isolate ASGS was selected and identified on the basis of citric acid production on rice substrate.

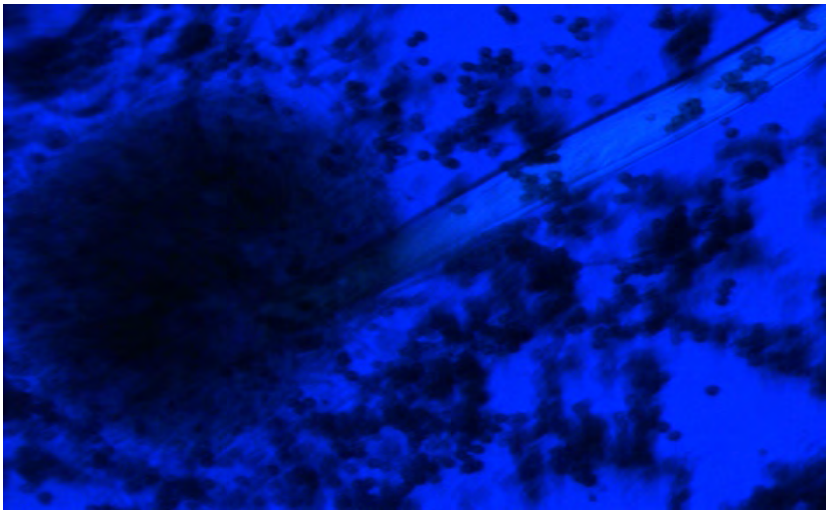
5.3.1 Cultural characteristics of the isolate ASGS

The isolate ASGS grown on potato dextrose agar as shown in figure 5 has black conidial areas and sparsely produced, after sub-culturing; many aspergilla were produced on PDA media. The colonies on the front side of PDA at 30ºc were wooly, initially white, quickly becoming black with conidial production whereas the reverse side of the plate showed white to pale yellow colour and growth produced radial fissures in the agar. It was found that the isolate ASGS was

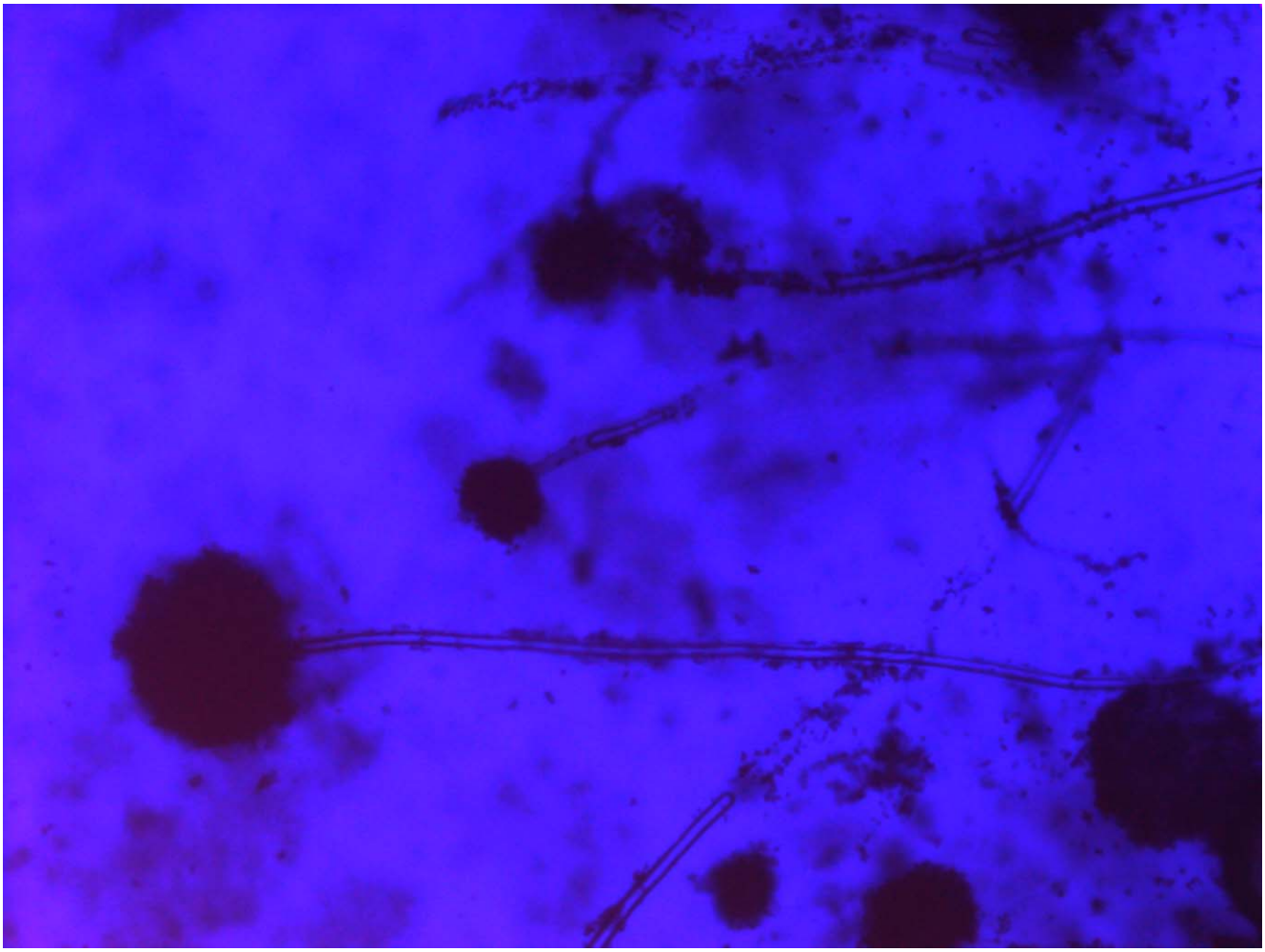
fast growing, covering the entire plate with in 2-3 days. At 30ºc, initial growth was white, which became black later and reverse turning pale yellow (Figure 6).

Figure 6. Colony appearance of the isolate ASGS, black conidial areas and reverse white to pale yellow colour.

Microscopic observation using lacto phenol aniline blue stain showed that the isolate ASGS had septate hyphae with smooth walled, simple conidiophores with conidial heads (Figure 7).



A



B

Figure 7. A and B, microscopic morphology of the isolate ASGS showing globose vesicle with biseriate, radiating head and dark, round conidia under phase contrast microscope AXIOSKOP40at(400x).

5.4 Citric acid production on different substrates

Shredded maize (M), wheat bran (W), Rice (R) and their different combinations showed different potential as a substrate for citric acid production (Figure 8). Of all the substrates which were tried for citric acid production, rice (R) was proved to be the maximum for citric acid fermentation (98g/kg DR) followed by WR (91g/kg DR), MR (78g/kg DR), and MW (65g/kg DR). The least citric acid was produced from MWR (20g/kg DR), M (13g/kg DR) and R (7g/kg

DR) substrates. All the experiments for screening of *A. niger* isolate and optimizing temperature, moisture, pH and duration parameters were conducted on rice solid substrate which was selected as a good fermentation medium.

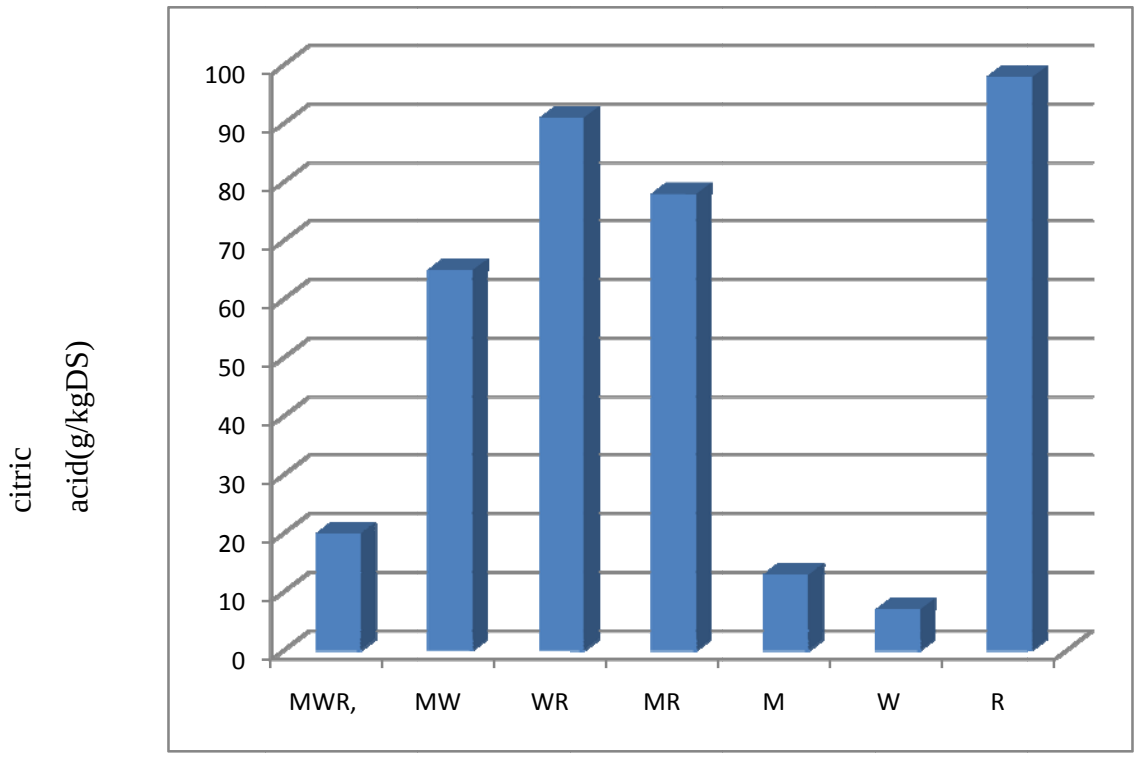


Figure 8. Comparison of MWR, MR, WR, MW, M, W and R substrates for citric acid production

Legends: MWR - shredded maize, wheat bran and rice

MR - shredded maize and rice M – shredded maize

WR - wheat bran and rice W – wheat bran

MW - shredded maize and wheat bran R – rice

5.5 Effect of moisture level on production of citric acid

Citric acid production was affected by the moisture content of the substrate. The highest citric acid production (91g/kg DR) was observed in rice-to-moisture ratio of 1:4. With increasing or

decreasing moisture level, citrate productivity was found to be decreased (Figure.9). The result showed that citric acid production was highest (91g/kg DR) at moisture level 80%.

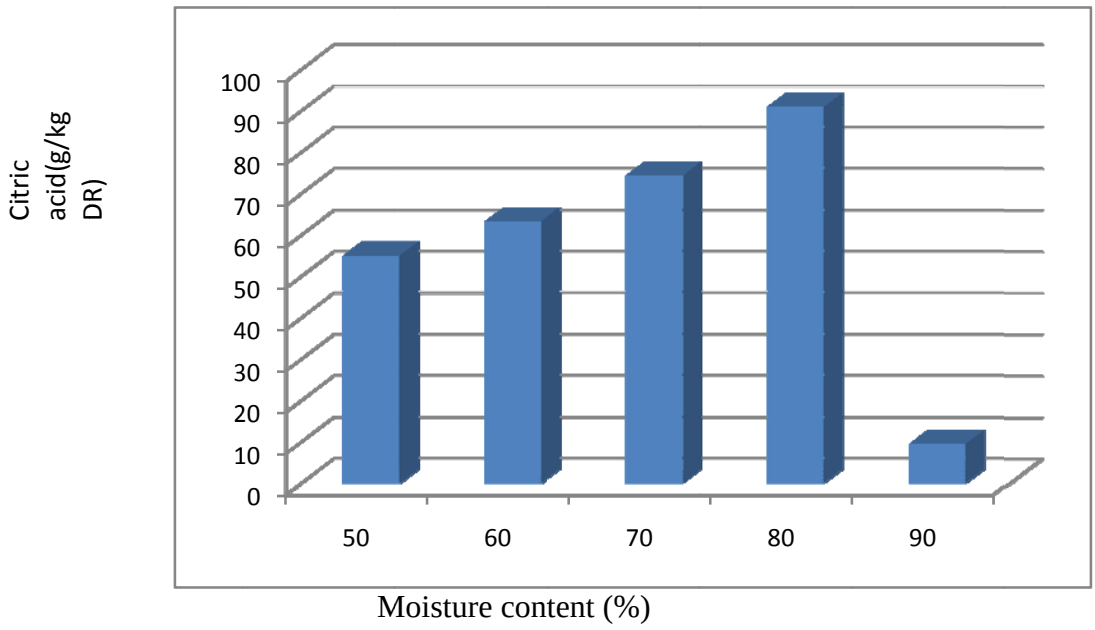


Figure 9 Effect of moisture content on citric acid production

5. 6 Effect of fermentation period on production of citric acid

Citric acid production was followed for a period of 144 hrs (6 days). Maximum citric acid production was observed at 72 hr (73.7g/kg DR). Further incubation after 72hrs showed a gradual decline in citric acid production (Figure 10).

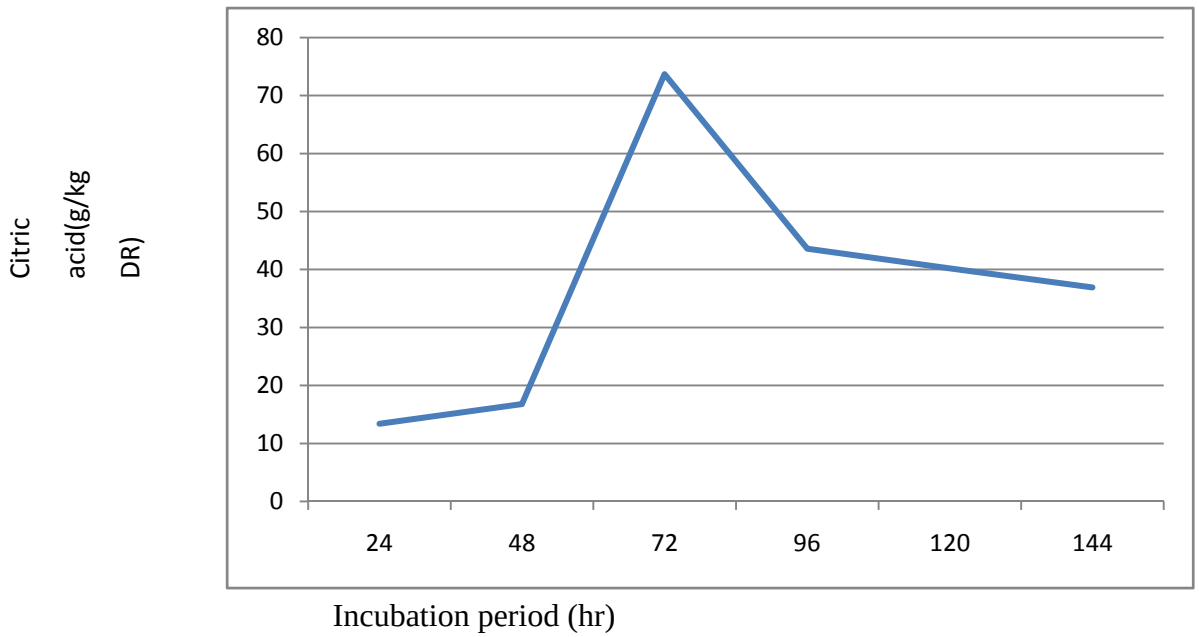


Figure 10: Time course of the citric acid production by *Aspergillus niger* isolate, ASGS on SSF.

5.7 Relative growth condition of the isolate ASGS on rice substrate at three incubation temperatures and different fermentation periods

The isolate was found to grow well at 30ºc and 35ºc. Maximum growth of white wrinkled mycelium was shown at 30ºc. Although good growth was shown at 35ºc, there was no white wrinkled mycelium, white powdery growth was observed at this temperature. Minimum growth was shown at 25ºc which became less powdery and white mycelium without wrinkled mycelial structure (Figure 11).

Legends: The first flask on the left side was incubated at 25ºc

The second and third flasks were incubated at 30ºc

The fourth flask(on the right side) was incubated at 35ºc

Figure 11. Growth of the isolate ASGS at three incubation temperatures and different fermentation periods.

Growth of the isolate at diferent fermentation periods as shown in table 7 was exponential with regard to biomass accumulation and complex morphology(white mycelium, yellow mycelial colour and formation of spores) was observed.

Table 7. Effect of duration on relative growth condition of the isolate ASGS at optimum temperature, 30°c

Strain	Duration				
	24hr	48hr	72hr	96hr	144hr
ASGS	+/-	+	++	+++	+++,Yellow color and sporulation

Key: +/-, not good ; +, good; ++, very good and +++, excellent.

Qualitatively, at the end of 72hr of fermentation periods, the isolate showed little yellow pigment. After 144hr of fermentation period, complete yellow pigment and sporulation was observed as shown in figure 12.

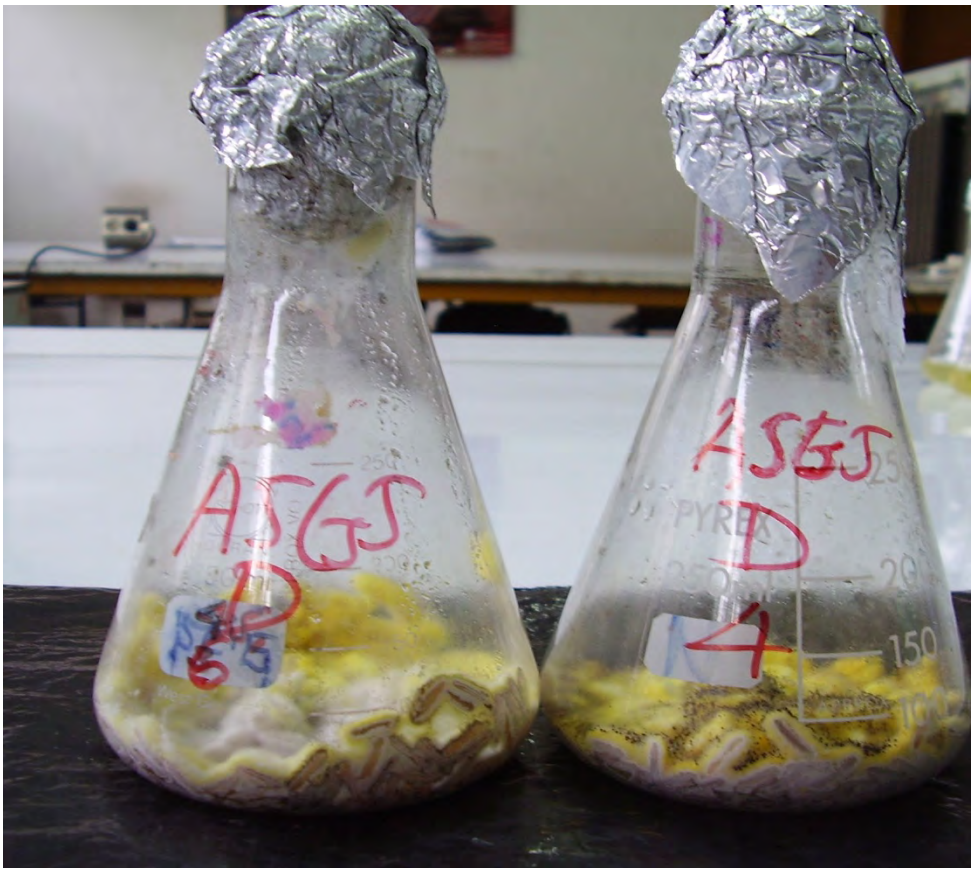


Figure 12. Appearance of yellow pigments after 72hr of incubation period

5. 8 Effect of Incubation temperature on production of citric acid

The citric acid accumulation increased significantly above a temperature of 25°c and production slightly declined at 35°c (Figure 13). The maximum citric acid (82.6g/kg DR) was produced by *A. niger* isolate (ASGS) at 30°c, whereas the lowest citric acid (59g/kg DR) production was obtained at 25°c.

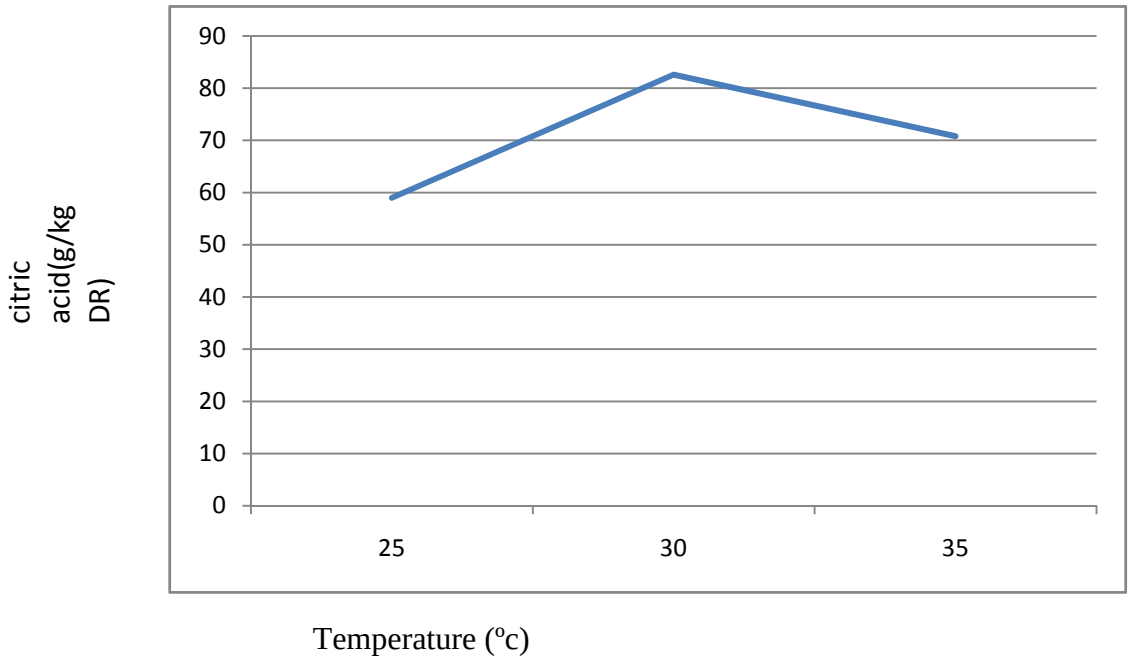


Figure 13. Effect of temperature on citric acid production

5. 9 Effect of pH on production of citric acid

Citrate production was measured at pH values 3.5, 4.5 and 5.5. High citric acid (98g/kg DR) was obtained at pH 4.5(Figure 14) which declined with an increase or decrease in pH at optimum temperature 30ºc.

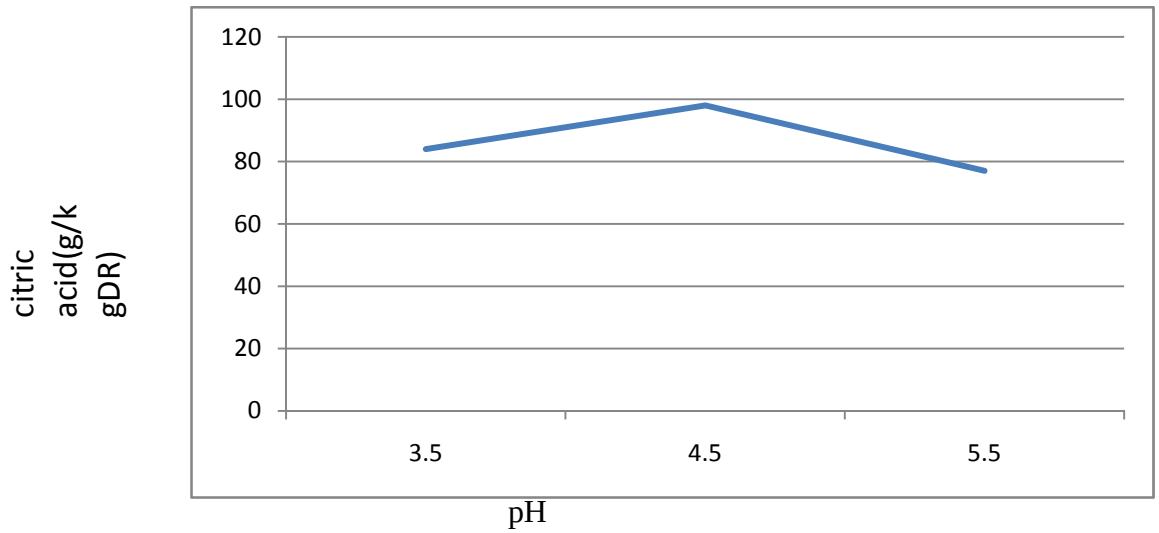


Figure 14: Effect of pH on production of citric acid

5.10 Recovery of citric acid from the fermented broth

Weight of the citric acid obtained from 10g rice after recovery was, W = 1.2g. Quantitative analysis of the product obtained was done photometrically by comparing it with the standard (Table 8). (Marrier and Boulet, 1958)

Table 8. Quantitative analysis of the recovered citric acid

	Optical density	Citric acid(g/l)
Standard(E. Merck, D-6100 Darmstadt, F. R. Germany)	0.09	51.1
Citric acid recovered	0.08	45.2
The difference was: 51.1-45.2 =5.9g/l		

Deviation was observed from the pure standard citric acid by 5.9g/l.

Table 10 showed that citric acid production varies depending up on the types of substrates and fermenters utilized. According to Rodrigues *et al*. (2008), the highest citric acid (616.5g/kg CP) was

produced from citric pulp using mutant strain of *A. niger* in Erlenmeyer flask. The lowest citric acid was produced from peat moss substrate using wild isolate of *A. niger* in flask level (Dhandayuthapani, 2009).

Table 9. Comparison of the result with the previous findings that were published by various scientists

Substrate	Fermenter	Citric acid(g/kg DS)	Reference
Cassava bagasse(CB)	Column incubator	309.5	Vandenberghe *et al*., 2000
Date pulp	Erlenmeyer flask	168	Assadi and Nikkhah, 2002
CB	Flask	220	Vandenberghe *et al*., 2003
CB	Column	309	Vandenberghe *et al*., 2003
CB	Tray	263	Vandenberghe *et al*., 2003
CB	Horizontal drum	269	Vandenberghe *et al*., 2003
Apple pomace	Flask	256	Ji-rong *et al*., 2003
Carob	Flask	30	Alani *et al*., 2007
Areca husk	Flask	119.42	Narayanamurthy *et al*., 2008
Citric pulp(CP)	Flask	616.5	Rodrigues *et al*.,2008
Peat Moss	Flask	27.5	Dhandayuthapani,2009
Rice	Flask	110.6	Present work

produced from mutant strain of *A. niger*

5.2 DISCUSSION

In this study, a total of five *A. niger* isolates, ASAS, ASBS, ASCS, ASGS and ASMS were isolated from different samples taken from soil, seed and environment. All isolates were similar morphologically when they were examined under microscope. All the isolates have black conidial areas and sparsely produced, after sub-culturing; many aspergilla produced on PDA media. The cultures in general, were fast growing, covering the entire plate with in 2-3 days. At 30^{o}c, initial growths were wooly, white, which became black later and reverse turning pale yellow colour (Figure 6).

In order to get higher citrate producing strain, all the five isolates were screened on rice solid substrate. Out of these, three strains, ASGS, ASBS and ASCS (60%) were found to produce higher citric acid on rice solid substrate of which 2/3 of them were obtained from seeds. The remaining two isolates, ASAS and ASMS were less effective. In general, the percentage of the isolates screened as higher citrate producers indicated that yet other effective strains could also be isolated from plants. As shown in the table 6, isolate ASGS was found to be more effective than the others, indicating that soil is a good habitat for industrially important microorganisms (Narayanamurthy *et al*., 2008) such as the one used in the present study.

Although inexpensive agricultural residues could be used as a solid substrate for citric acid fermentation (Prado *et al*., 2005), for the purpose of the present study, rice, maize and wheat bran were used. Out of the three substrates, rice(R) was found to be better citric acid producer followed by the combination of wheat bran and rice (WR). The combinations of maize and rice (MR); maize and wheat bran (MW) also showed the potential to be use as good substrate for citric acid production (Figure 8). The weakest substrate for citrate fermentation was wheat bran. According to Kumar *et al*. (2003), agglomeration in wheat bran causes non-uniform mixing of the substrate, which affects heat and mass transfer along with growth and product formation.

When the isolate ASGS was grown on rice at different incubation temperature, significant differences were observed. There was limited growth and biomass formation at 25°c; this may be

due to inactivation of enzyme system. Though maximum growth was shown at 30°c, the isolate also showed growth and biomass accumulation at 35°c (Figure 11).

Biomass production of the isolate during the fermentation process was not pronounced within 24-48hrs (Table 7). However, increase in growth was detected after 48hr which culminated in excellent growth after 96hrs of incubation indicating that the isolate take some time to establish during the fermentation process.

As illustrated in the figure 10, citrate accumulation increased exponentially after 48hrs of fermentation and the maximum citric acid (73.7g/kg DR) was obtained at 72hr. Similarly, Narayanamurthy *et al*. (2008) reported that maximum citric acid was obtained after three days of fermentation. According to Kristiansen and Sinclair (1979) decline in concentration of citric acid may have been due to decay in the enzyme systems responsible for the production of citric acid up on exhaustion of fermentable sugars, or could be attributed to citric acid break down due to the toxic effect of accumulated waste product, Roukas (1999).

Initial rice moisture content had a considerable effect on citric acid production by ASGS as illustrated in Figure 9. A maximum citric acid production of 91g/kg DR was obtained with 80% moisture content. An initial moisture content dropping from 70% resulted in a drop in citric acid production. According to Nampoothiri *et al.* (2003), if the moisture content of the substrate is too low, it generally results in a low nutrient solubility and poor substrate swelling, reducing citric acid production. If it also exceeds the substrate's retention capacity, it results in insufficient aeration and less mycelium growth (Battaglin *et al*., 1991).

As illustrated in the figure 13, ASGS produced a higher level of citric acid at 30°C. Citrate accumulation increased significantly with increase in temperature from 25 to 30°c and production slightly declined beyond 30°c. This was due to denaturation of enzyme system of *A.niger* at higher temperature (Bajpai and Margaritis, 1987). The result of the present study was in agreement with those of Narayanamurthy *et al*. (2008) who studied areca husk for citric acid production by *Aspergillus niger* under SSF. Nampoothiri *et al*. (2003) found that citric acid

production could be affected by a retarded germination of fungi, slow metabolic activity, enzyme denaturation and low cell viability. However, at 35°c also, higher amount of citric acid (70.8g/kg DR) was produced. In this case, the present work is in line with the work of Parra *et al*. (2004) who studied about growth and spore production of wild type and genetically engineered *Aspergillus niger* strains. Temperature above 35ºC was inhibitory to citric acid formation because of the increased production of byproduct acids and also inhibition of culture development (Ikram-ul-Haq *et al*., 2002).

As shown in the figure 14, the citric acid concentration increased with an increase of pH up to 4.5 and decreased beyond this value. The highest value of citric acid concentration (98g/kg DR) was achieved at an initial pH of 4.5, indicating that the optimum pH is 4.5.

Table 8 showed that the deviation of the recovered citric acid from the pure standard citric acid. As the impurities in the recovered acid decreased its concentration, the spectroscopic absorbance was less, indicating that the recovered citric acid was slightly less than the standard in concentration.

The summary of the previous findings on table 9 showed that there is variation on citric acid production using *Aspergillus niger*. The type of substrate, fermenter and type of strain used are among the essential conditions for citric acid production. The highest citric acid (616.5g/kg DS) was produced from citrus pulp using mutant strain of *A.niger* indicating that gene modification of wild isolate is important for commercial production of citric acid.

6. CONCLUTIONS AND RECOMMENDATIONS

6.1 Conclusions

In the present study, effective citric acid producing *Aspergillus niger* isolates were recovered from different soil, seed and environment. On the basis of the production of the high concentration of citric acid, one isolate ASGS (soil) was selected and further used to optimize citric acid production with various nutritional and environmental factors.

The optimization experiments showed that rice is a good substrate, with moisture content 80%. The maximum was at growth temperature of 30ºc, with the maximum citric acid production (110.6g/kg DR) at incubation period of 72hr, and pH adjusted to 4.5.

6.2 Recommendations

- Isolation and screening of effective citrate producing strains from various sources such as soil, seed and air is possible and this should be further investigated to get the best citrate producer strain.
- Further study on optimization of parameters such as temperature, moisture, pH and nutrients for high citric acid production also needed.
- As the present work and the previous findings showed, citric acid production on solid state fermentation is possible.

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