BIOCONVERSION OF MOLASSES TO CITRIC ACID

A THESIS

Submitted in Fulfilment of the

Requirements for the Award of the Degree

OF

DOCTOR OF PHILOSOPHY

By

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"Beauty lies in the eyes of the beholder"
Beautiful ———— is Aspergillus niger!

Candidate's Declaration

I hereby certify that the work which is being presented in this thesis entitled **BIOCONVERSION OF MOLASSES TO CITRIC ACID** in fulfilment of the requirements for the award of the Degree of **DOCTOR OF PHILOSOPHY,** submitted in the Department of Biosciences & Biotechnology of the University is an authentic record of my work carried out during a period from September, 1986 to November, 1990 under the supervision of Prof. C.B. Sharma and Dr. B.M.J. Pereira, Department of Biosciences & Biotechnology, University of Roorkee, Roorkee.

The matter embodied in this thesis has not been submitted by me for the award of any other degree.

Kush Garg

Date : 28.11.90 **(KUSH GARG)**

This is to certify that the above statement made by the candidate is correct to the best of our knowledge.

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ABSTRACT

India produces a huge quantity of sugarcane molasses every year as a by-product of the sugar industry, which can be used as a potential source of substrate for the production of citric acid through fermentation using *Aspergillus niger* . However, to accomplish this, there are two major requirements that must be fulfilled. Firstly, the sugarcane molasses contains some inhibitory substances, including heavy metal ions (Fe^{3+}, Cu^{2+}, Zn^{2+} and Mn^{2+}) which should be removed prior to using it as substrate for citric acid fermentation. Secondly, availability of a high citric acid yielding strain of *A. niger* which would be less susceptible to heavy metal toxicity. The first requirement was met by treating the diluted molasses, containing 12% sugars, with 0.1% hexacyanoferrate (HCF) at pH 4.5 and 70-90°C for 15 min. After this pretreatment, molasses was found to be a better substrate for citric acid fermentation. For the second requirement, the wild type *A. niger* cells were subjected to a two-stage UV mutagenesis resulting in a high citric acid-yielding mutant (KCU520) of *A. niger* which was isolated and characterized. The mutant cells were found to be morphologically distinct. These were white in colour, short and thick with branches compared to yellow colour, thin and smooth structures of the wild type. The mutant cells were easily maintained in culture without any loss of efficiency in citric acid production. Optimum conditions for citric acid production by *A. niger* KCU520 were as follows: pH, 4.5; temperature, 28°C; sugar concentration, 12% and fermentation period, 10 days. Under these conditions 60%, 75% and 80-85% sugars were converted to citric acid in submerged, surface and solid state surface fermentation (SSF) systems, respectively. Various regulators, such as 2% groundnut oil, 0.1% H_2O_2 or 0.3% starch in the fermentation medium were also found to enhance the citric acid yield by 5-10%. In the case of SSF, the microbial bed grown on the solid surface could be reused, at least in three subsequent fermentation cycles with fresh medium, with equal efficiency. In fact, in the 2nd and 3rd cycle, maximum yield was achieved in 5 days. The SSF process was scaled-up to 10 liters without loss of efficiency. Similar results were obtained when Ca- alginate immobilized cells were used for fermentation. It was found that *A. niger* KCU520 cells require a 6 day acclimatization period after which these can be used repeatedly in 4 fermentation cycles, each giving maximum yield in 6-7 days. The polyacrylamide gel immobilization of *A. niger* KCU520 cells was also tried in a two-stage bioreactor. After a 6 day acclimatization period under optimum conditions of fermentation, these immobilized cells were found to convert about 20% sugars into citric acid in 24 h and the cells remained viable for 3 to 4 weeks without any significant loss of activity. Hence, by using immobilized cells in a two-stage bioreactor it was possible to obtain a continuous production of citric acid from sugarcane molasses at the rate of 20 g/lit/day. Scaling-up of this procedure is in progress.

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First I bow down at the lotus feet of **Maa Saraswati** and pray, "Let noble thoughts come to me from all the universe and by the eternal blessings of Omniscient, Almighty as to enable persist always in me the strong belief of devotion and determination that anything incomprehensible on this holy earth is comprehensible through team-work with the spirit of cooperation, goodwill and hardwork".

In this scientific and technological society of superexcellence and eminence with a high intellectual fervour, apart from personal efforts and steadfastness to work, persistent inspiration and constant encouragement of countless number of individuals comming from different sections of academic achievements serve as the driving force. To quote them all may not quite aptly suit the bill; although the taboo of conciseness does not spare the vastness, but would definitely convey the intensity of feelings of one's heart in acknowledging their encouragement and assistance with kindest regards.

In Hindu Mythology, gurus are respected as "**Sakshat Parambramha**". In Sanskrit, it is quoted as;

गुरुर्ब्रह्मा गुरुर्विष्णु गुरुर्देवो महेश्वरः ।
गुरुर् साक्षात् परम् ब्रह्म् तस्मै श्रीगुरवे नमः ।।

(In Hindu Mythology, Teachers are worshipped as God Brahma (God of Creation), God Vishnu (God of Protection), and God Maheshwara (God of Destruction); Guru is Omniscient, therefore, I bow down to my respected Guides)

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In this strenuous task of my doctoral research, I received directly and indirectly assistance and help from my innumerable colleagues. To acknowledge their whole-hearted assistance, I virtually fall short of words; therefore, **I am leaving this acknowledgment incomplete ..... in their reminiscences.**

Kush Garg

(KUSH GARG)

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ABBREVIATIONS USED

ATCC - American Type Culture Collection
AU - Acid Unitage
^{o}C - ^{o}Centigrade
cf. - Cross Reference
cm - Centimeter
Conc. - Concentration
dO - Dissolved Oxygen
DOT - Dissolved Oxygen Tension
Fig. - Figure
g - Gram
h - Hour
HCF - Hexacyanoferrate
IWC - Immobilized Whole Cell
KCB - Kush; Chandra; Biosciences
KCU - Kush; Chandra; Ultraviolet
l/lit - Litre
mg - Milligram
min - Minute
mm - Millimetre
ug - Microgram
ul - Microlitre
N - Nitrogen
N - Normality
NADH -Nicotinamide Adenine Dinucleotide
nm - Nanometer
P - Phosphate
PAG - Polyacrylamide Gel
PDA - Potato Dextrose Agar
ppm - Parts per Million
rpm - Revolutions per Minute
SEM - Scanning Electron Microscopy
SSF - Solid State Surface Fermentation
TEMED- N-N-N'-N'-Tetramethyl ethyl diamine
UV - Ultra Violet
Var. - Variety
v/v - Volume/Volume
W - Watt
wt - Weight
w/v - Weight/Volume

1.0 INTRODUCTION

Citric acid is one of the bulk chemicals used by the food and pharmaceutical industries. In nature it is formed as an intermediate in tricarboxylic acid cycle (Fig.1). Citrus fruit juice is a rich natural source of citric acid, and in the early years of the twentieth century citric acid was commercially isolated from lemon juice. By the middle of the 20th century, fermentation processes for citric acid production on a commercial scale were developed using sugarbeet molasses and hydrocarbons as substrates and fungal cells as microbes. *Aspergillus niger* cells were extensively used for citric acid fermentation from sugarbeet molasses. However, the fermentation technology for citric acid has to date been a well guarded secret.

India, being a major sugarcane producing country, produces a huge quantity of sugarcane molasses every year which is used in an uneconomical manner for the production of cheap quality alcohol. Infact, a large quantity of molasses is just wasted without any proper use. Unfortunately, no serious attempt has so far been made to develop an indigenous technology for citric acid production based on sugarcane molasses, inspite of the fact that a major portion of the country's need for citric acid is met by imports.

Realizing the need for an indigenous technology for citric acid production based on local resources, the present study was undertaken. The sugarcane molasses obtained from local sugarcane industries was used in the research and development work. Our approach to the problem, which in fact constituted the major objectives of the work presented in this thesis, was as follows:

(i) To obtain, by selection and mutagenesis, a high citric acid yielding strain of *Aspergillus niger* , a commonly used fungus for citric acid fermentation from molasses.

(ii) To develop a cheap method for clarification of sugarcane molasses which is known to contain some inhibitory substances, including excess of heavy metal ions (Cu^{2+}, Zn^{2+}, Mn^{2+} and Fe^{3+}).

(iii) To develop a suitable fermentation technology for large scale production of citric acid from molasses.

FIG.1 METABOLIC REGULATION OF CITRIC ACID ACCUMULATION IN *Aspergillus niger*. (Rohr et al., 1983).
(+) Activation and (-) Inhibition.

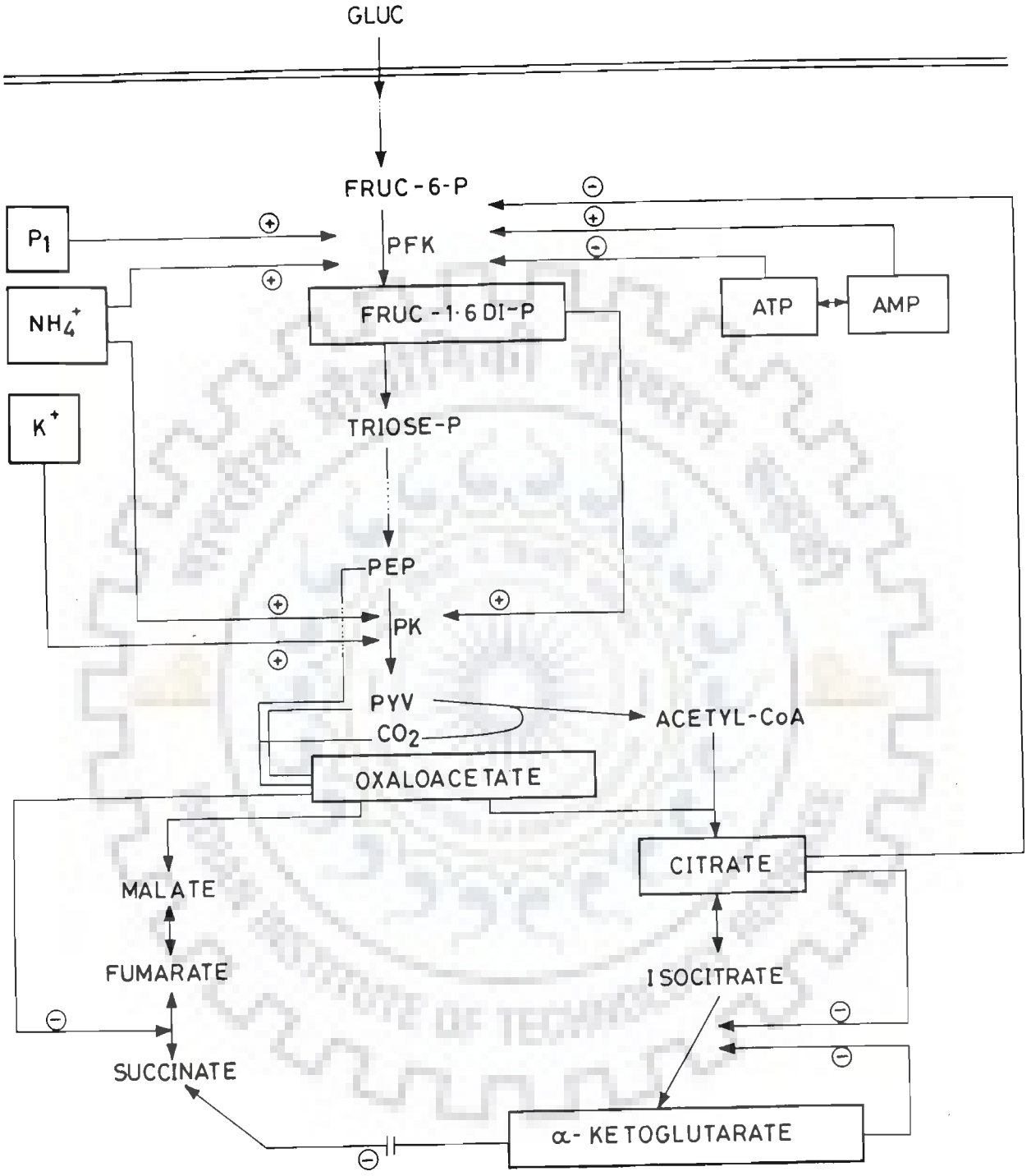
GLUC
FRUC-6-P
P1
PFK
NH4+
FRUC-1·6 DI-P
ATP
AMP
K+
TRIOSE-P
PEP
PK
PYV
CO2
ACETYL-CoA
OXALOACETATE
CITRATE
MALATE
FUMARATE
ISOCITRATE
SUCCINATE
α-KETOGLUTARATE

Fig. 1

(iv) **To develop and design a bioreactor for continuous or semi- continuous production of citric acid using immobilized cells.**

This thesis embodies the findings and conclusions on the above mentioned aspects of citric acid fermentation with sugarcane molasses as a substrate.

2.0 LITERATURE REVIEW

2.1 HISTORICAL BACKGROUND

Citric acid, 2-hydroxy-1,2,3,-propentricarboxylic acid, was first isolated from lemon juice by Scheele in 1784. It was, however, produced commercially by John & Edmund Sturge (England) in 1826 using calcium citrate which was derived from lemon juice. Later, this method of citric acid manufacture was also used in America, France and Germany. But due to high cost and uncertain supply of calcium citrate, other methods of citric acid manufacture were sought. As a result, the fermentation process for citric acid manufacture was developed and the commercial plants based on fermentation technology were started in Belgium (1919), USA (1923) and UK (1928), followed by USSR, Czechoslovakia and Germany.

Since that time, considerable efforts have been made towards the elucidation of the microbial and biochemical fundamentals as well as towards the improvement of fermentation process for the citric acid production. An account of the early work is given by Bernhauer (1940), Foster (1949), Johnson (1954) and Prescott and Dunn (1959). The recent developments on citric acid fermentation technology have been reviewed by Perlman and Sih (1960), Lockwood (1975), Berry et al. (1977), Kapoor et al. (1982), Rohr et al. (1983), Milsom and Meers (1985) and Kubicek and Rohr (1986). In the beginning, citric acid was produced using various members of genus *Penicillium* (Thom and Currie, 1916) with some high yielding *Penicillium* strains - *Penicillium janthinellum* Biourge Var. *Kuensanii* and *P. restrictum* Gilman and Abbott Var. *Kuensanii* (cf. Rohr et al.,1983). Since citric acid fermentation with *Penicillium* was done at neutral pH, contamination was a serious problem. Hence, it was replaced by *Aspergillius* genus, which was found to produce appreciable amount of citric acid at low pH (Currie, 1917).

Various species of *Aspergillus* are reported for citric acid production. Apart from *Aspergillus niger*, which is most widely used, other species like *A. awamori, A. clavatus, A. fenicis, A. fonsecaeus, A. fumaricus, A. luchensis, A. saitoi, A. usumii* and *A. wenti* were described, but never tried on commercial scale (cf. Perlman and Sih, 1960; Rohr et al., 1983).

Apart from *Aspergillus* species, other fungi were also known to excrete citric acid. These include, *Trichoderma viride, Botrytis cinerea, Ustulina vulgaris, Ascochyta, Absidia, Talaromyces, Acremonium, Eupencillium* and *Mucor piriformis*. Of these, only *T. viride* was studied in some detail. Certain yeasts, mainly of the genus *Candida* were also used for production of citric acid from hydrocarbons. The most commonly used yeast strains capable of utilizing n-alkanes as carbon source are : *C. lypolytica, C. tropicalis, C. guilliermondii, C. intermedia, C. parapsilosis* and *C. oleophila* (cf. Kapoor et al., 1982; Rohr et al., 1983).

Between 1966 and 1967, several of the well-known favourite mutants of Coryneform bacteria, such as; *Corynebacterium*, *Arthrobactor* and *Brevibacterium*, were found to produce citric acid from n-paraffins (C_9 to C_{30}) and similar substrates (cf. Rohr et al., 1983).

2.2 FERMENTATION MEDIUM

Shu and Johnson (1948a) presented the first systematic study of the influence of medium composition on citric acid production. It was observed that in addition to traces of minor elements, the media should contain phosphate, magnesium and potassium salts (Currie, 1917; Doelger and Prescott, 1934; Perquin, 1938; Wells and Herrick, 1938; Von Loesecke, 1945; Shu and Johnson, 1948a; Martin and Steel, 1955). Moreover, by adjusting the concentration of salts carefully, it was possible to achieve more citric acid accumulation with a negligible amount of oxalic acid, which otherwise is produced alongwith citric acid in significant amounts during fermentation.

2.2.1 Carbon source

For industrial scale production of citric acid, a cheap and readily assimilable carbohydrate source is a primary requirement. Various carbohydrate sources have been used. For instance, beet molasses, cane molasses, unrefined sucrose, citrus molasses, cassava root and starch cake, cane juice, wheat starch, sago starch, sweet potato starch, tapioca, etc. (cf. Perlman and Sih, 1960). In addition, the possibility of using cotton waste (Kiel et al., 1981), apple pomace (Hang and Woodams, 1984), grape pomace (Hang and Woodams, 1985), wood hemicellulose (Maddox et al., 1985) and cellulose hydrolysate (Monomani and Sreekantiah, 1987) were also explored.

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However, of the above mentioned substrates, only molasses is used widely on a commercial scale. In Europe and other countries beet molasses is used for citric acid production, and the technology is a guarded secret. However, in India, beet molasses is not readily available. In contrast, cane molasses is produced in huge amounts as a by-product of sugar industries, hence is a potential substrate for citric acid production. But cane molasses was found to be a very complex system for citric acid production (Karow and Waksman, 1947; Tressl et al., 1976) by *Aspergillus niger*. Perlman and Sih (1960) stated that in addition to the problem of metal ion contents, there are several other inhibitory substances, e.g., acetic acid and certain peptides in cane molasses, which must be removed prior to citric acid fermentation. On the other hand, some stimulators in cane molasses were also reported although their chemical nature has not been elucidated (Leopold and Valtr, 1961; Fencl and Vintrova, 1963; Ilczuk, 1967; Janota-Bassalik et al., 1970).

2.2.2 Nitrogen

Generally, nitrogen is supplied either in the form of ammonium nitrate or sulphate, in the fermentation medium. The effect of different nitrogen source on citric acid fermentation was also studied (Bernhauer, 1928). Normally the NH_4^+ ion concentration is kept in the range of 0.3 to 1.5 g/l. It was found that concentration of NH_4NO_3 greater than 0.25% , leads to the accumulation of oxalic acid (Naguchi and Bando, 1960; Gupta et al., 1976). Rohr and Kubicek (1981) reported the importance of NH_4^+ as an enzymic regulator for citric acid accumulation. NH_4^+ was found to be a potent positive regulator for phosphofructokinase enzyme, the key regulatory enzyme for citric acid accumulation.

2.2.3 Phosphate

It is well established that phosphate is an essential requirement for fungal growth. However, in order to achieve citric acid accumulation, the fungal growth must be restricted. Firstly, Perquin (1938) showed the citric acid overflow due to phosphate deficiency in the medium. Later, Szucs (1944) reported that in presence of assimilable phosphorus, submerged citric acid fermentation is not possible. A number of investigators, employing surface culture method, used phosphate deficiency to bring about citric acid accumulation

(cf. Johnson, 1954). However, Shu and Johnson (1947; 48a; 48b) showed that phosphate alone is not the critical factor, but rather the balance between manganese, zinc and phosphate. Martin and Steel (1955) reported the formation of certain sugar acids in the presence of high concentration of phosphorus.

2.2.4 Trace metals

Trace metals are very important for citric acid accumulation due to their opposite effect at low and high concentrations. It was established that the growth requirements of *A. niger* include carbon, hydrogen, oxygen, nitrogen, potassium, phosphorus, magnesium and sulphur (Foster, 1939). The literature contains seemingly conflicting reports regarding the influence of Zn, Fe, Cu and Mn on the citric acid production by *A. niger* (Von Loesecke, 1945). Steinberg (1935), who was primarily concerned with the nutrition of fungus rather than the acid production, found that Zn, Fe, Cu and Mn were necessary for the development of low pH in the medium, but this low pH was not reached if Mn was present in amounts optimum for growth. Currie (1917), believed Fe to be unnecessary for citric acid production and found that Zn increases the weight of mycelium but not acid. Porges (1932) and Shu and Johnson (1948b) employed Zn and Fe in their media in order to obtain high yields of citric acid. Perlman et al. (1946) used Fe in media containing very low impurities of Zn, Cu and Mn, and obtained high acid yields, but higher concentration of Zn or Cu was found to be inhibitory. Shu and Johnson (1948b) found that Mn in very low concentration caused marked decrease in the yield of acid. Porges (1932) reported that Mn and Cu were inhibitory for citric acid production. Tomlinson et al.(1950; 51) confirmed that Fe, Zn, Cu and Mn in distinctly low concentration are all essential for citric acid accumulation. Noyes (1969) reported the possibility of eliminating the negative effect of Fe with high concentration of Cu. The critical nature of Mn was also shown by Clark et al. (1966). They also reported that from a number of trace metals added, the Mn seems to have more drastic effect on citric acid yield than Fe and Zn.

The physiological and metabolic changes in *A. niger*, grown in citric acid producing medium under Mn deficiency, were also worked out (Kubicek and Rohr, 1977; Kubicek and Rohr, 1978; Kubicek et al., 1979; Kisser et al., 1980; Ma et al., 1985). In general, Mn deficiency reduces the activity of pentose phosphate pathway and the activity of tricarboxylic acid cycle enzymes and also results in some impairment of anabolism. Most important is the

elevated intracellular pool of NH_4^+ which is very likely to result from a disturbance in protein turnover thereby causing an increased breakdown of protein (Kubicek et al., 1979). This high intracellular concentration of NH_4^+ could be a very potent regulator of phosphofructokinase since it has been shown to relieve phosphofructokinase from inhibition by citrate.

2.2.5 Aeration

Industrial production of various metabolites by filamentous fungi is susceptible to regulation by dissolved oxygen tension (DOT) of the medium. In case of citric acid production by *A. niger,* the influence of oxygen has been well established (Martin and Waters, 1952; Shu, 1953; Steel et al., 1954; Steel et al., 1955; Martin, 1957; Clark and Lentz, 1961), but the metabolic basis of this phenomenon was not investigated.

Citric acid production is stimulated by increase in aeration. It can even further be increased by supplementing pure oxygen instead of air. Clark and Lentz (1961) showed that the air can be recirculated after removing CO_2. If air supply of the medium was stopped for some time during the fermentation phase, product formation was limited or fully stopped inspite of the resumption of aeration (Batti, 1966; Kovats and Sackowska, 1976). Kubicek et al. (1980) showed that a high DOT is essential for citric acid production.

Kubicek and Rohr (1980) gave a biochemical interpretation of regulatory role of O_2 on citric acid accumulation by suggesting that oxygen is used as one of the substrates which is metabolically needed for the reoxidation of glycolytic NADH.

2.2.6 pH

The main reason for the failure of citric acid fermentation using *Penicillium* sp. was microbial contamination at neutral pH. Fundamental investigations by Thom and Currie (1916) on citric acid producing properties of *Aspergilli* revealed that *A. niger* can grow well at pH values around 2.5 to 3.5 and citric acid was abundantly produced at pH values even lower than 2.0. However, using molasses as carbohydrate source, an initial pH of 4.0 to 6.0

due to the presence of acetic acid (Fencl and Leopold, 1957; 59). When decatanized molasses was used, the initial pH of the medium could be maintained between 1.4-2.8 (Miles Lab., 1962).

2.2.7 Temperature

Importance of incubation temperature during citric acid production was emphasized by many workers (Doelger and Prescott, 1934; Kovats, 1946; Tomlinson et al., 1951; Kitos et al., 1953). A temperature of 28-30°C has been shown to give high yield and also rapid rate of accumulation of citric acid (Prescott and Dunn, 1959), although at a lower temperature (20°C) the contamination by other acids was reduced to minimal (Kitos et al., 1953; Martin, 1957; Chmiel, 1975a). Tomlinson et al. (1951) showed that temperature affects the metal ion resistance of fungus. Kovats (1946) advocated a two-stage process in which germination was done at 28-30°C, while rest of the fermentation was done at 20°C for getting higher citric acid yields. Perlman (1947) showed that increasing the temperature of a culture from 25° to 37°C results in rapid utilization of the remaining sucrose.

2.2.8 Additives and stimulants

Effect of various regulators on citric acid production has been reported in literature. Millis et al. (1963) established that fatty materials, specially those containing a high proportion of unsaturated fatty acids, greatly enhanced the citric acid yield. Gold and Kieber (1967) reported that use of small quantities of starch is beneficial for citric acid fermentation. Bruchmann (1966) reported that by addition of small quantities of H_2O_2 in fermentation medium, a high citric acid yield was achieved. The mechanism by which the citric acid formation is affected by these regulators is not very clear.

The effect of other chemicals with no nutritional value in improving citric acid production has also been examined. For example, inhibitors of metabolism such as CaF, NaF, KF have been found to accelerate citric acid production. Similarly, malic hydrazide, under submerged culture conditions, has been found to increase citric acid production by *A. niger*. Other chemical additives such as aromatic amides, esters of dichloroacetic acid, sodium sulphite and crysyllic acids, were found to be protecting against the contamination by other

micro- organisms. In addition, various complex components such as mycelial digests, Baker's yeast, rice bran, etc., were also found to be useful in increasing citric acid yields (cf. Kapoor et al., 1982).

2.3 COMMERCIAL PRODUCTION PROCEDURE

The detailed know-how of citric acid fermentation is a well guarded secret by the manufacturers and only a surface knowledge is seen in the literature (Foster, 1949; Johnson, 1954; Prescott and Dunn, 1959; Perlman and Sih, 1960; Lockwood and Schweiger, 1967; Lockwood, 1975; Kapoor et al., 1982; Rohr et al., 1983; Milsom and Meers, 1985). Most commonly, the citric acid fermentation is essentially done by cultivating *A. niger* fungus, either in the traditional surface fermentation or batch or feed batch submerged fermentation, using a molasses or sucrose based medium. The success of the fermentation process depends upon the quality of the feed-stock and the microbial strain. Different manufacturers have developed their own microbial strains for the feed-stock available to them.

2.3.1 Industrial strains

Since most of the work regarding strain improvement is done in industrial research and development, much information regarding production strain is not yet available. Many laboratories employed Wisconsin strains 72-4 (identified with NRC A-1-123) also available as ATCC 11 414 (Shu and Johnson, 1948a). In central Europe, many strains may be traced back to research activity at Kaznejow and Prague (Bernhauer, 1940).

Although for an efficient industrial process a potent strain is an absolute necessity, surprisingly very little work has been done in the field of strain improvement. Perlman (1947) investigated some seventy five strains of *A. niger* for their ability to produce citric acid in submerged culture. He found considerable variation among them and reported some metal resistant strains. Exposure of spores to ultra- violet light (Gardner et al., 1956; Das and Nandi, 1965; Das and Roy, 1981), X-rays (Quilico et al., 1949; Diller et al., 1950; Gardner et al., 1956), hertzian waves, radium salts, gamma rays (Das and Nandi, 1969; Islam et al., 1984; 1986), or certain chemicals (Das and Nandi, 1969; Das and Roy, 1981) results in mutations, and a few mutants were found to have improved acid producing

capacity as compared to the parent culture (cf. Perlman and Sih, 1960; Das, 1972; Rohr et al., 1983). Two or more stage mutagenesis and combined treatment by physical and chemical mutagenic agents has been found to be more advantageous than the use of a single mutagen to improve citric acid production in *A. niger* (cf. Berry et al., 1977). Various auxotrophic mutants of *A. niger* have been also screened by various workers without much success (Das, 1980; Muliskova et al., 1980). Polyploidy and heterokinesis for better citric acid yield was also tried (Das and Roy, 1978). The occurrence of parasexual hybridization in *A. niger* was also used as an alternative technique for obtaining active strains for citric acid production (cf. Berry et al., 1977). Recently Kirimura et al. (1986) developed diploid strains of *A. niger* by protoplast fusion technique for citric acid production. They have also improved the citric acid yield by haploidization of diploid *A. niger* (Kirimura et al., 1988).

The technique of induced mutation and strain selection has now been accepted as a routine practice to get improved microbial strains for industrial production. Obviously, this is a time consuming and laborious process as it requires screening of several thousand colonies to get a mutant of improved potency. Although there are some observations to correlate high citric acid production, with such characters like, culture morphology (Doelger and Prescott, 1934; Gardner et al., 1956; Shcherbakova, 1963; Das and Nandi, 1965) anatomical structures (Protod'yakonov and Kresling, 1935), mycelium fat contents (kresling, 1936), etc., the process also involves random use of mutagens to get a large population of desired characters.

Johnston (1974), stressed on the need for frequent isolation of high yielding mutants, since upon longer storage the conidial material shows 'degeneration'. Previously, single spore technique for screening of mutants was used. Later, James et al. (1956) improved the previous existing technique of Foster and Davis (1949) and Quilico et al. (1949) by incorporating pH indicator in the agar medium. Rohr et al. (1979) further improved this method by direct conidial analysis in the first place, by the application of Ehrlich's reagent, a specific reagent for citric acid detection.

2.3.2 Inoculum development

Like strain selection, spore propagation for actual application in fermentation is also a very important part of production strategy. There can be two possible strategies for the

preparation of inoculum; either by suspending spores in the liquid sporulation medium (Martin and Waters, 1952; Hustede and Rudy, 1976b) or harvesting in dry form (Waller, 1946). An entirely different strategy for inoculum preparation was given by Foster (1949) by harvesting spores on a disintegrated support material like bran, soaked with appropriate sporulation medium.

Doelger and Prescott (1934) noted that successive transfer of the culture on a synthetic medium had a great effect on citric acid production in submerged cultures. However, Bernhauer et al. (1941) found that sub-culturing, repeatedly in 'synthetic media', often leads to degeneration. Shu and Johnson (1947) studied the effect of sporulation medium on citric acid yields. In submerged fermentation, morphology of mycelium and also the shape of hyphae of inoculum is very important for citric acid fermentation. It was found that hyphae, when inoculated to fermenter, should be abnormally short, stubby, forked and bulbous (Snell and Schweiger, 1951; Hustede and Rudy, 1976a; Kisser et al., 1980). This stage of affairs is brought about by a deficiency of Mn in the medium (Kisser et al., 1980) or is obviously related to addition of ferrocyanide ions (Hustede and Rudy, 1976a). Factors leading to such a stage are; correct ferrocyanide level (Clark, 1964; Hustede and Rudy, 1976b), an iron concentration of less than 1 ppm (Snell and Schweiger, 1951), adjustment of pH (Fried and Sandza, 1959), adjustment of aeration and agitation (Svenska Sockerfabriks, 1964), concentration of Mn (Clark et al., 1966) and amount of spore inoculum (Berry et al., 1977). Recently, Gomez et al. (1988) showed the effect of pellet growth on citric acid production.

2.3.3 Raw materials

As stated earlier, various cheap carbohydrate sources can be utilized for citric acid production. However, molasses has found wide-spread use for citric acid production. The principal reasons for this are its low price, compared to other sources of sugars, and presence of various essential nutrients besides sucrose. These include minerals, organic compounds and vitamins (Olbrich, 1963). Mainly, beet and sugarcane molasses are used for citric acid production by *A. niger*. However, molasses represents a complex system. Various problems were faced in its efficient utilization for citric acid fermentation (cf. Perlman and Sih, 1960; Kapoor et al., 1982). One of the major problems is the variation in its composition and mineral contents. Hence, molasses of different origins give different

yield of citric acid (Tressl et al., 1976). There seems to be involved more than one factor in the regulation of citric acid yield during fermentation. In any case, it would be necessary to evaluate the stability of the stock-material and also to standardize the method of pretreatment of molasses, especially the clarification procedure used to reduce or eliminate trace metal concentration in molasses.

2.3.4 Clarification of substrate

The problem of clarification of carbohydrate source is very old and was realized as soon as the first factory for citric acid was erected in Belgium in 1919. It was in the factory of Kaznejow that hexacyanoferrate (HCF) was first used as a means of precipitating unwanted metals in molasses solution, but this was apparently kept secret (cf. Rohr et al., 1983). Clark (1962) and Clark et al. (1965; 66), had done a complete study on the effect of HCF on molasses medium. They showed that by HCF treatment most of metals, known to interfere with citric acid production, were efficiently precipitated without affecting nitrogen, carbon and phosphorus contents of the medium.

Several other methods were used to remove contaminating metallic ions from the fermentation media including treatment with aluminium hydroxide and passage of medium over ion exchange resins. Addition to the media of chelating agents or specific precipitants including morphiline, quaternary ammonium or cyclic ammonium compounds, small amount of milk powder, camphor or tannic acid, gelatin, EDTA, activated charcoal, polyethylene amine, were also found to give higher yield of citric acid (cf. Perlman and Sih, 1960; Kapoor et al., 1982).

The effect of lower alcohols, especially methanol, on citric acid production is of great interest. It is most widely used clarification method with the exception of Cu addition and HCF precipitation. Moyer (1953a, 1953b) showed that addition of various lower alcohols in small concentrations reversed the negative effect of trace metals on citric acid fermentation.

2.3.5 Fermentation process

Three types of fermentation processes are generally used for the manufacture of citric acid. These are Japanese Koji or solid state fermentation process, the liquid surface culture fermentation, and the submerged fermentation process (Foster, 1949; Johnson, 1954; Prescott and Dunn, 1959; Perlman and Sih, 1960; Lockwood and Schweiger, 1967; Lockwood, 1975; Berry et al., 1977; Kapoor et al., 1982; Rohr et al., 1983). Recently, use of the immobilized whole cells is being investigated for continuous production of citric acid (Eikmeier and Rehm, 1984; Horitsu et al., 1985; Tsay and To, 1987; Chung and Chang, 1988).

2.3.5.1 Solid state fermentation process

The term solid state fermentation is described as any fermentation process in which the substrate is not a free liquid. Cahn (1935) first showed the citric acid production by solid state fermentation, solid material such as cane or beet pulp was impregnated with sugar solutions like sucrose or molasses. Later, Lakshminarayana et al. (1975) and Chaudhary et al. (1978) reported higher yields of citric acid by solid state fermentation technique using sugar free sugarcane bagasse as solid support.

It is a relatively simple process for citric acid production and was first used for industrial production of citric acid in Japan. The raw materials used for this process were starch containing residual pulps from starch manufacture, e.g., sweet potatoes, fibrous residues, rice or wheat bran and others. Lockwood (1975) estimated total citric acid production by SSF as being about 2,500 tons per year.

2.3.5.2 Surface fermentation process

In the classical process for the manufacture of citric acid, the culture medium is kept in shallow pans and the fungus develops as a mycelium mat on the surface of medium. This type of process is termed as 'surface or shallow pan process'. In this process, the cost of production of citric acid is low. It is still used by many manufacturers, but few detailed

descriptions are available (British Intell. Obj. Sub-Committee, 1946; Mallea, 1950; Johnson, 1954).

The system consists of a fermenter in which shallow trays are stacked one over the other and an effective air circulation system by which humidified air is circulated in a laminar-flow manner. The medium is filled in these trays while hot (cf. Rohr et al., 1983). See Ref 136 The medium can be inoculated either by liquid spore suspension or by spraying spores through air circulation. In the presence of trace metals, formation of a yellow pigment, 'asperenone' occurs which is excreted to the medium and is difficult to remove (Jefferson, 1967). This phenomenon indicates a faulty operation.

2.3.5.3 Submerged fermentation process

The advantages of the submerged fermentation process over the liquid surface fermentation process in terms of investment and operating cost induced investigators to attempt and develop a submerged fermentation process for citric acid production. Amelung (1930), using a black *Aspergillus,* which he called *A. niger japonicus,* slowly bubbled stream of air through a culture solution. He obtained sub-surface growth and found citric acid in the culture solution. Kluyver and Perquin (1933) described the use of cultures on shaker, a technique for the study of submerged fermentation on small scale. Perquin (1938), used shaker cultures for the study of the production of organic acids. Szucs (1944) obtained a patent for the submerged culture manufacture of citric acid.

Both conventional stirred reactors and tower fermenters are being used for this process. The characteristic of inoculum pellet during formation is an important factor which governs citric acid yield (Whitaker and Long, 1973; Gomez et al., 1988). The advantage of the submerged fermentation lies in the fact that it can be in continuous operation for citric acid production (Kristiansen and Charley, 1981).

2.3.5.4 Immobilized cell fermentation process

Recently, immobilized biocatalyst technology has rapidly emerged as a novel means to utilize enzymes as efficient and heterogeneous catalysts for a multitude of industrial and

medical applications (Abbot, 1976; Chibata and Tosa, 1977; Jack and Zajic, 1977; Brodelius, 1978; Chibata, 1978; Klien and Wagner, 1978; Linko, 1980; Linko and Linko, 1984). Physical entrapment, in porous polymeric carriers, is by far the most widely used technique for whole cell immobilization. The classical system is the free radical initiated co-polymerization of acrylamide and bis-acrylamide, which was the first successful attempt in whole cell immobilization by entrapment (Mosbach and Mosbach, 1966). The same system was used in the first successful industrial application of an immobilized cell process (Chibata et al., 1974). While this technology has been replaced by other convenient methods, it is still a preferred method for initial laboratory scale studies. There are only few reports regarding citric acid production from immobilized cells in polyacrylamide gel (Vieth and Venkatasubramanian 1978; Horitsu et al., 1985). Much work remains to be done for its use in large scale production of citric acid.

The application of alginate for the purpose of whole cell immobilization was first reported in 1975 (Hackel et al., 1975). Later, Kierstan and Bucke (1977) modified the previous method by using Ca-alginate combination. The immobilization of living microbes in Ca-alginate is now widely used, since the method is very simple and also the gel material is non-toxic and cheap (Kierstan and Bucke, 1977; Ohlson et al., 1979; Vaija et al., 1982). Regarding citric acid production from *A. niger* by immobilization in Ca-alginate beads, there are only a few reports in literature. Veith and Venkatasubramanian (1976) and Vaija et al. (1982) entrapped the mycelia of *A. niger* with Ca-alginate and hide collagen for citric acid production. Henrich and Rehm (1982) attempted immobilization by absorption of mycelia onto a glass-carrier in a fixed-bed reactor and studied citric acid fermentation. Later, citric acid producing properties and morphological developments of *A. niger*, immobilized in Ca-alginate beads were reported by many workers (Eikmeier et al., 1984; Eikmeier and Rehm, 1984; Tsay and To, 1987).

2.3.5 Product recovery

To obtain the desired product from the fermented broth in a final form is one of the most important part of any fermentation industry. For citric acid recovery, first, mycelium is to be separated from the broth and then citric acid is extracted either by classical calcium oxide precipitation method or by solvent extraction method.

The classical citric acid recovery process is particularly suitable for use with very impure liquors derived from molasses. Solvent extraction is a possible alternative of the classical method, but since this procedure also extracts some of the impurities present in molasses derived liquors, it is easier to apply it to the products from glucose or alkane based substrates (cf. Milsom and Meers, 1985). Various solvents (such as, hydrocarbons, e.g., octane, benzene or kerosene; esters, e.g., n-butylacetate; ethers, e.g., n- or isobutylether; ketones, e.g., methylisobutyl ketone, etc.) have been used by several workers for this purpose (de Melle, 1963; Baniel et al., 1976; Wennerston, 1980; Alter and Blumberg, 1981).

3.0 MATERIALS AND METHODS

3.1 MATERIALS

Aspergillus niger KCU520 used in this study was obtained by UV mutagenesis of a locally isolated strain of *A. niger* (KCB1). It was maintained on potato dextrose agar (2.0% dextrose, 2.5% agar, in potato extract) slants, stored at 4°C and renewed every month.

Sugarcane molasses was obtained from the Mahalakshmi Sugar Mill Co. Ltd., Iqbalpur, Saharanpur (U.P.), India. All other chemicals were purchased from standard commercial manufacturers and were of the highest grade available.

3.2 METHODS

3.2.1 Strain selection

3.2.1.1 Isolation of strain

The parental *Aspergillus niger* (wild type) strain was isolated from a biogas digester. The digester effluent was centrifuged at 2,000 rpm and the clear supernatant was transferred onto a yeast extract agar medium (Glucose, 10 g/l; Yeast extract, 10 g/l; Agar, 15 g/l; NaCl, 1 g/l; $MgSO_4.7H_2O$, 0.25 g/l; $FeSO_4.7H_2O$, 0.01 g/l). These Petriplates were incubated at 22°C for 10 days. Various types of fungi which were grown on the plates were isolated and transferred onto separate PDA slants and incubated at 30°C for 5 days. Of these, 5 colonies of *Aspergillus niger* (KCB1-5) were isolated and examined for citric acid fermentation. Preliminary fermentation trials showed that *A. niger* KCB1 was the most promising strain. It was referred to as the parent strain and used in further mutagenesis experiments. The culture was maintained on PDA slants, stored at 4°C and renewed every other month.

3.2.1.2 Mutagenenis and screening

Mutagenesis and screening of the mutagenized colonies was done according to the method of Rohr et al. (1979), by using the filter paper culture technique and employing p-dimethylaminobenzaldehyde for accurate on the spot detection of citric acid.

Paper discs (circular sheets of 2 mm chromatographic paper and 120 mm diameter) were placed in glass Petriplates and sterilized. After cooling, each was soaked with 23 ml of sterilized medium. To each paper disc 0.5 ul conidial suspension in saline, of the strength of $2x10^3$ conidia/ml (containing nearly one conidium), was applied with the help of a fine capillary tube (0.7 mm diameter). 15-20 spots were applied onto each paper disc and then subjected to mutagenic treatment. Mutagenic treatment was carried out with ultraviolet irradiation (260 nm) using 15 W UV tube (Phillips) from a distance of 50 cm. Samples were irradiated with UV for 10-60 minutes and then stored in dark at 4°C overnight to avoid photorepair and were then passed through the routine procedure of screening as described below.

After mutagenesis, each filter paper culture plate was incubated for 115 h at 30°C. After incubation, replica plates for each treatment were prepared on the PDA culture medium. The original paper discs were dried for two hours at 80°C and citric acid was assayed by spraying the plates with 4% (w/v) p-dimethylaminobenzaldehyde in acetic anhydride followed by heating the paper for 1 min at 140°C. Citric acid zones were indicated by the purple coloured spots thus obtained on the paper disc. This is an extremely sensitive method and as low as 1 ug of citric acid can be detected on the paper without any interference of mineral or other organic acids commonly produced by *A. niger*. The citric acid producing capacity (Acid Unitage value or AU value) of different colonies was determined by dividing the diameter of the citric acid zone with the diameter of colonies. Citric acid fermentation was also carried out using *A. niger* mutants, cultured on PDA replica plates, and fermentation yield was determined.

3.2.2 Fermentation medium

Preliminary studies of citric acid fermentation optimization were carried out using Doelger and Prescott medium (1934). After optimizing the major nutrients requirement for citric acid fermentation, all studies were performed on a medium composed of the following components :

Sucrose	-	**14% or molasses, containing 10-12% total sugars**
NH_4NO_3	-	**0.25%**
K_2HPO_4	-	**0.1%**
$MgSO_4.7H_2O$	-	**0.03%**

The pH of the medium was adjusted to 2.0 (4.0-4.5 in case of molasses). The medium was then autoclaved at 121°C for 15 min at 15 psi.. Whenever the effect of citric acid regulators was investigated, these were added to the fermentation medium in appropriate concentrations before sterilization.

3.2.3 Clarification of molasses

Since crude molasses was found to contain a high concentration of heavy metals and other compounds, which were inhibitory for citric acid fermentation, the crude molasses was subjected to clarification process.

The crude cane molasses was diluted 4-5 times with deionized water and varying concentrations (0.5-2.0 g/l) of potassium ferrocyanide (HCF) were added followed by heating at 70-90°C for 15 min at pH 4.0-4.5. The precipitate, containing metal complex salt, was removed by filtration through Whatman 41 filter paper. The filtrate was referred to as clarified molasses. After adjusting to pH 4.5 with 1N HCl, the clarified molasses was used for preparation of fermentation medium.

3.2.4 Inoculum

Aspergillus niger from slants was subcultured on to Petriplates, containing nearly 15 ml of PDA medium and incubated at 30°C for 5 days. The spores were aseptically harvested in sterile distilled water. A 1-2% v/v inoculum, containing nearly 10^6 - 10^7 spores/ml, was used to inoculate culture flasks containing fermentation medium. In order to study the effect of spore age on the citric acid production, incubations were performed for different periods and samples were tested at each stage of growth.

3.2.5 Citric acid fermentation by free *Aspergillus niger*

3.2.5.1 Submerged fermentation process

Fifty ml of fermentation medium in 250 ml conical flasks were inoculated with *A. niger* and incubated at 28°C in an orbital shaker with constant shaking of 150 rpm. The fermentation was carried out for 10-12 days. After completion of fermentation, the media was autoclaved, biomass was separated by filtration under suction and the fermented broth was analyzed for total acids and citric acid.

3.2.5.2 Surface fermentation process

In surface fermentation process, after inoculating the medium, the culture flasks were kept in an incubator at 28°C. No agitation or shaking was done and the organism was allowed to grow on the surface of the medium in the form of a mycelial mat. When needed, water suction pump was used to remove waste fermentation gases from the fermentation flask and also to circulate the air (1.5-2.0 l/min) over the mycelial mat. After fermentation, cultures were autoclaved, biomass was separated by filtration and the broth was tested for both total acids and citric acid.

3.2.5.3 Solid-state surface fermentation (SSF) process

Solid state surface fermentation (SSF) was performed for citric acid production by using bagasse as solid support. Before use, the finely powdered bagasse (obtained from a sugar mill) was treated with 2N HCl at room temperature overnight, followed by thorough washing with distilled water until the washing was neutral.

A slurry of medium with bagasse, containing normally 15% bagasse (70% moisture contents), was first sterilized and then inoculated with *A. niger* spores. The culture flasks were kept in an incubator at 28°C, without shaking, to ensure a surface culture. Waste fermentation gases were removed by connecting the fermentation flasks to the water suction pump. This also provided circulation of air over the solid surface. After the fermentation had reached the desired state, it was terminated by autoclaving, mycelium mat was separated and the broth was analyzed for both total acids and citric acid.

SSF was also carried out in a replacement batch process by supplementing the fresh medium after exhaustion of nutrients. This was done by cultivating fungus on a solid support of cane bagasse mixed with molasses medium in a 250 ml conical flask, with an outlet/inlet at the bottom, through which fermented medium was removed and fresh medium refilled, under aseptic conditions (Fig.2). The process is as follows :

The medium mixed with 15% bagasse (70% moisture contents) was inoculated with appropriate amount of *A. niger* KCU520 spores. The spores were allowed to grow on the medium for a desired period. Following the time periods for growth, the fermentation medium was drained out and replaced by fresh medium. The citric acid contents were monitored at each stage of fermentation. In addition, the acidity was tested at 24 h intervals by withdrawing 1 ml of broth from the fermentation flask and when further production of citric acid was stopped, the old medium containing citric acid produced during the fermentation was removed and a fresh batch of sterilized medium was added. This cycle of replacement of old medium by the fresh medium was continued until the citric acid production was substantial.

FIG.2 MULTIPLE CYCLE SOLID STATE SURFACE FERMENTATION IN FLASKS.

Note the mycelium grown on the surface of the medium to form a thick mat for surface fermentation. After the desired intervals, fermented broth was withdrawn and fresh medium introduced into the flasks through an inlet/outlet provided at the bottom of the flask. The fermentation was carried out in an incubator at 28°C without shaking.

* Top panel shows the fermentation assembly without the aid of an aeration facility.

* Bottom panel shows the fermentation assembly with proper aeration facility.For aeration, 8 mm glass tube, packed with sterilized glass wool, was inserted in the fermentation flask through the cotton plug and connected to the water suction pump which sucks the waste fermentation gases and automatically helps in fresh air circulation to the culture flask by sucking air through the cotton plug.

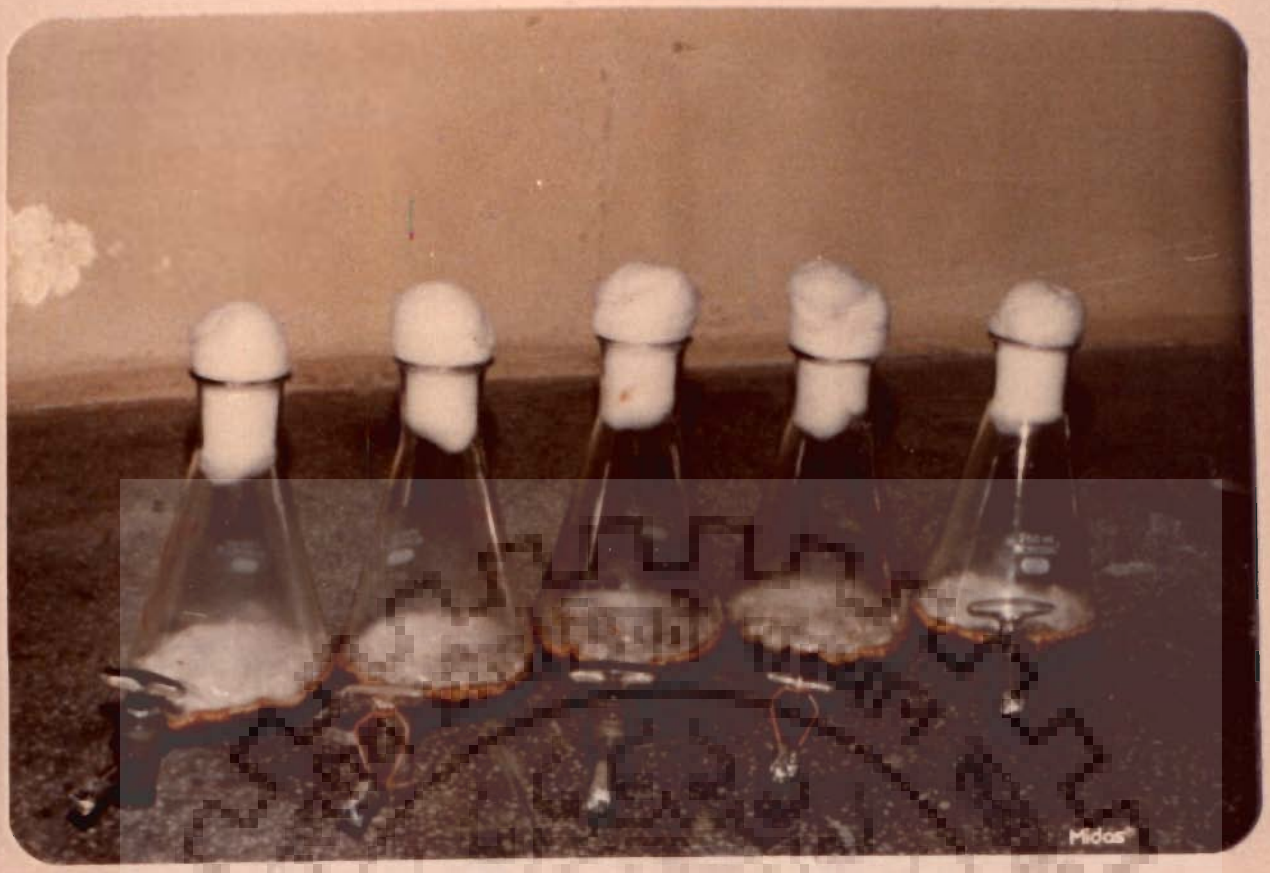


Fig.2

3.2.5.3.1 Scaling-up of solid state surface fermentation

A large scale solid state surface fermenter was also designed to study scaling-up of citric acid fermentation. The fermentation was done in circular stainless steel plates (30x30x3 cm) fitted in an incubator (Fig.3). Each plate contained 1 litre of medium mixed with 150 g of bagasse (70% moisture contents). Humidified air was passed through a sterile glass-wool filter and then circulated inside the fermenter, over the top of the cultures with the help of an air compressor.

3.2.6 Citric acid fermentation by immobilized whole cells of *Aspergillus niger*

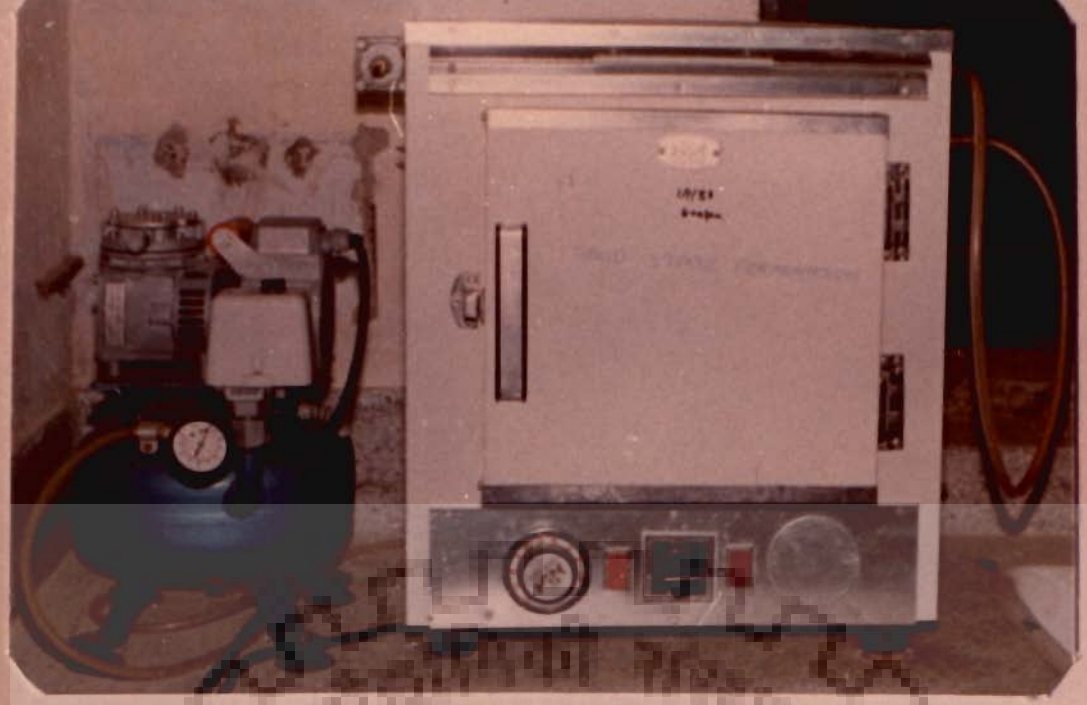
Citric acid fermentation was also studied with immobilized *A. niger*. Polyacrylamide gel and sodium alginate entrapment methods were used for immobilization of fungus.

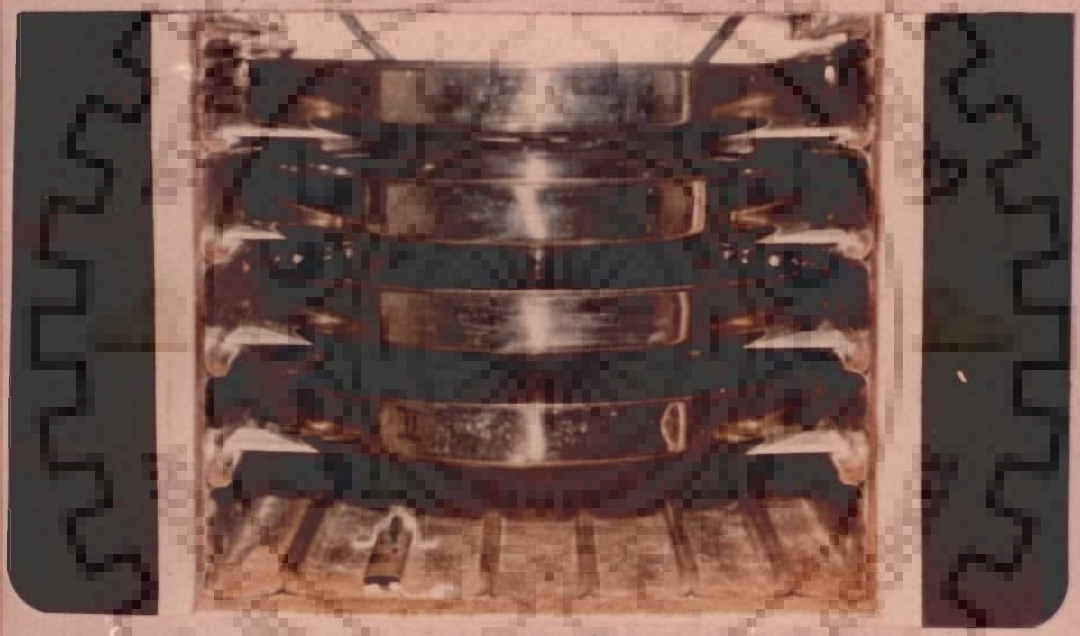
3.2.6.1 Immobilization of A. niger by sodium alginate entrapment

A. niger KCU520 from slants was subcultured onto Petriplates and incubated at 30°C for 5-days. The spores were aseptically harvested in sterile distilled water (10^7-10^8 spores/ml). To 400 ml of 8% Na- alginate solution, previously autoclaved at 110°C for 10 min and cooled to room temperature, was added 600 ml of spore suspension containing nearly 10^7-10^8 spores/ml. The contents were mixed thoroughly and the mixture was poured, drop by drop, into 1% sterilized solution of $CaCl_2$ solution with the help of an injection syringe, at 10°C, under aseptic conditions. The beads thus formed were allowed to harden for an hour at 10°C. Beads were then washed thoroughly with 0.9% NaCl solution and stored at 4°C before use.

FIG.3 AN INDIGENOUS SURFACE FERMENTER.

A surface fermenter consisting of an oil free compressor (left), an incubator to hold stainless steel trays (30x30x3 cm) and an air circulation system for horizontal air flow over each tray. The incubator is provided with temperature control from 20° to 240°C. Before use, the unit is sterilized by maintaining the incubator temperature at 150°C for 1 h. Top panel shows the exterior of the fermenter showing the oil free compressor (left) and the incubator (right) ; middle panel shows stainless steel trays used for the surface fermentation and ; bottom panel shows the perforated copper tube through which sterilized air was circulated over the surface of the mycelium bed in each tray.





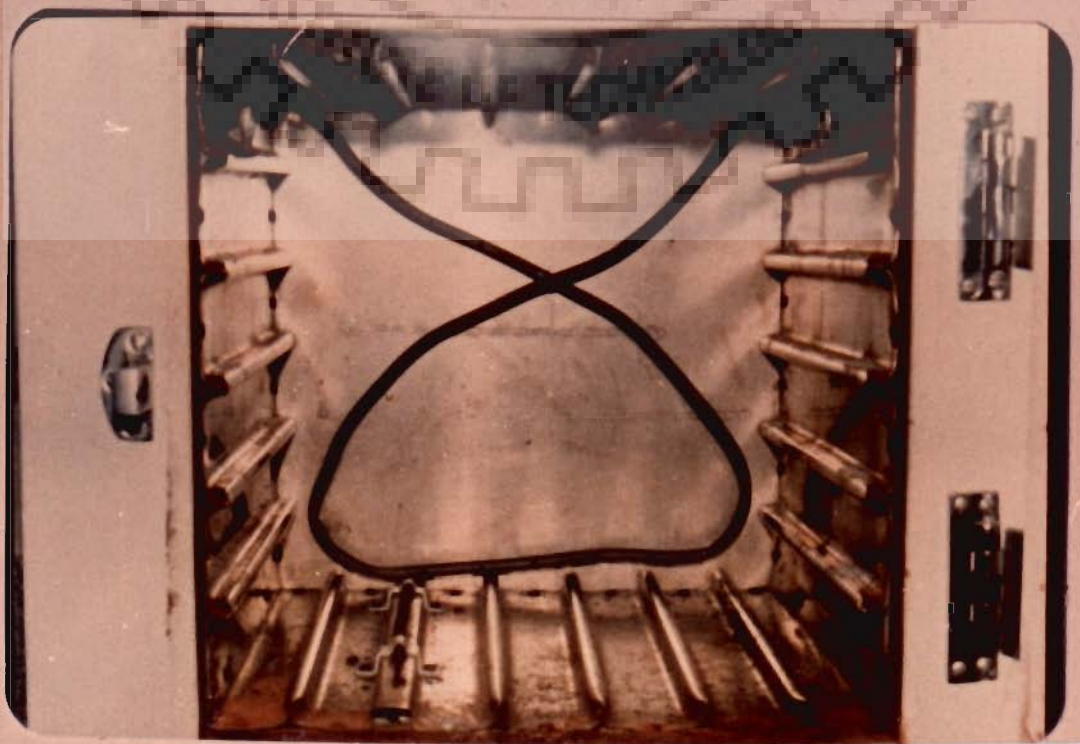


Fig.3

3.2.6.1.1 Fermentation with alginate immobilized cells of *A. niger*

3.2.6.1.1.1 Batch fermentation :

Conical flasks (250 ml capacity), containing nearly 50 ml of fermentation medium (pH 4.0), were charged with 100 (approximately 10 g) immobilized conidia beads. These flasks were incubated at 30°C in an orbital shaker at 150 rpm for 15 days or for desired periods.

3.2.6.1.1.2 Replacement batch fermentation :

Replacement batch fermentation was done in a single-stage bioreactor (Fig.4). For fermentation, the bioreactor was charged with 20 g of beads in 100 ml of fermentation medium. The fermentation was carried out under highly aerated conditions at 32°C. First replacement was done after 6 days of fermentation in which whole fermented broth was replaced with fresh medium and the fermented medium was analyzed for citric acid yield. Further replacements were done when no appreciable increase in citric acid yield was observed with time.

3.2.6.2 Immobilization by polyacrylamide gel (PAG) entrapment

In order to produce more biomass of *A. niger* KCU520 for immobilization, spores ($5x10^7$) were inoculated into 100 ml of culture medium, composed of sugarcane molasses (10% total carbohydrates, pH 5.5), in a 500 ml conical flask and cultured in an orbital shaker at 30°C at 150 rpm. The growing cells were harvested after 96 h incubation period by centrifugation at 7,000 rpm for 15 min and washed thoroughly with sterilized distilled water under aseptic conditions. Approximately, 20 g of wet cells were suspended in 64 ml of 0.9% NaCl solution and were incubated at 4°C for 24 h before immobilization. To this cold solution were added 12 g of acrylamide, 0.64 g bis- acrylamide, 8.0 ml of 5% TEMED and 8.0 ml of 2.5% ammonium per sulfate. The reaction mixture was shaken well, poured into a glass tray (25x15x5 cm) to give a thickness of 1 mm and allowed to polymerize at 10°C.

After the gel formation, 4x4x1 mm slice, containing immobilized cells was cut, washed twice with saline and stored at 10°C in saline solution before use.

3.2.6.2.1 Fermentation with PAG immobilized cells of *A. niger*

3.2.6.2.1.1 Use of single-stage bioreactor

The immobilized *A. niger* KCU520 cells were loosely packed in an indigenously designed bioreactor (Fig.4) which was connected to a water suction pump through a side arm containing sterilized glass wool to circulate air into the fermenter (2 l/min). Fermentation was carried-out using the replacement method described above and the citric acid produced was monitored every 24 h. The suction of the sterilized air from the bottom of the reactor also provided an automatic stirring and mixing of the immobilized cells with the medium.

3.2.6.2.1.2 Use of a two-stage bioreactor

A two-stage bioreactor was also designed, which is essentially a combination of two single-stage bioreactors in series (Fig.5). The working of this system is the same as that of the single-stage bioreactor. For fermentation, nearly 20 g of gel, containing immobilized fungus, was suspended in 100 ml of medium in the Ist column of the two-stage bioreactor. The incubation was done under high aeration rates (2-3 l/min) at 35°C (unless specified otherwise). The medium was replaced with fresh medium after every 24 h under aseptic conditions. After 24 h fermentation period in the Ist column of the bioreactor, medium of the first compartment was passed to the second compartment containing 20 g immobilized cells for another 24 h incubation at 35°C. To the Ist column, 100 ml medium was added and fermentation continued as usual for 24 h. In this way a continuous 2 cycle replacement for citric acid fermentation was achieved, which definitely improved the yield considerably.

FIG.4 SINGLE-STAGE BIOREACTOR FOR IMMOBILIZED CELL FERMENTATION.

A glass bioreactor of 400 cm^3 (20x5 cm) showing connection for suction pump used to remove waste gases evolved during fermentation process and to supply sterilized air through the side arm containing thick pads of glass-wool, to the bioreactor. The fermentation liquid was withdrawn from the outlet provided at the bottom of the bioreactor.

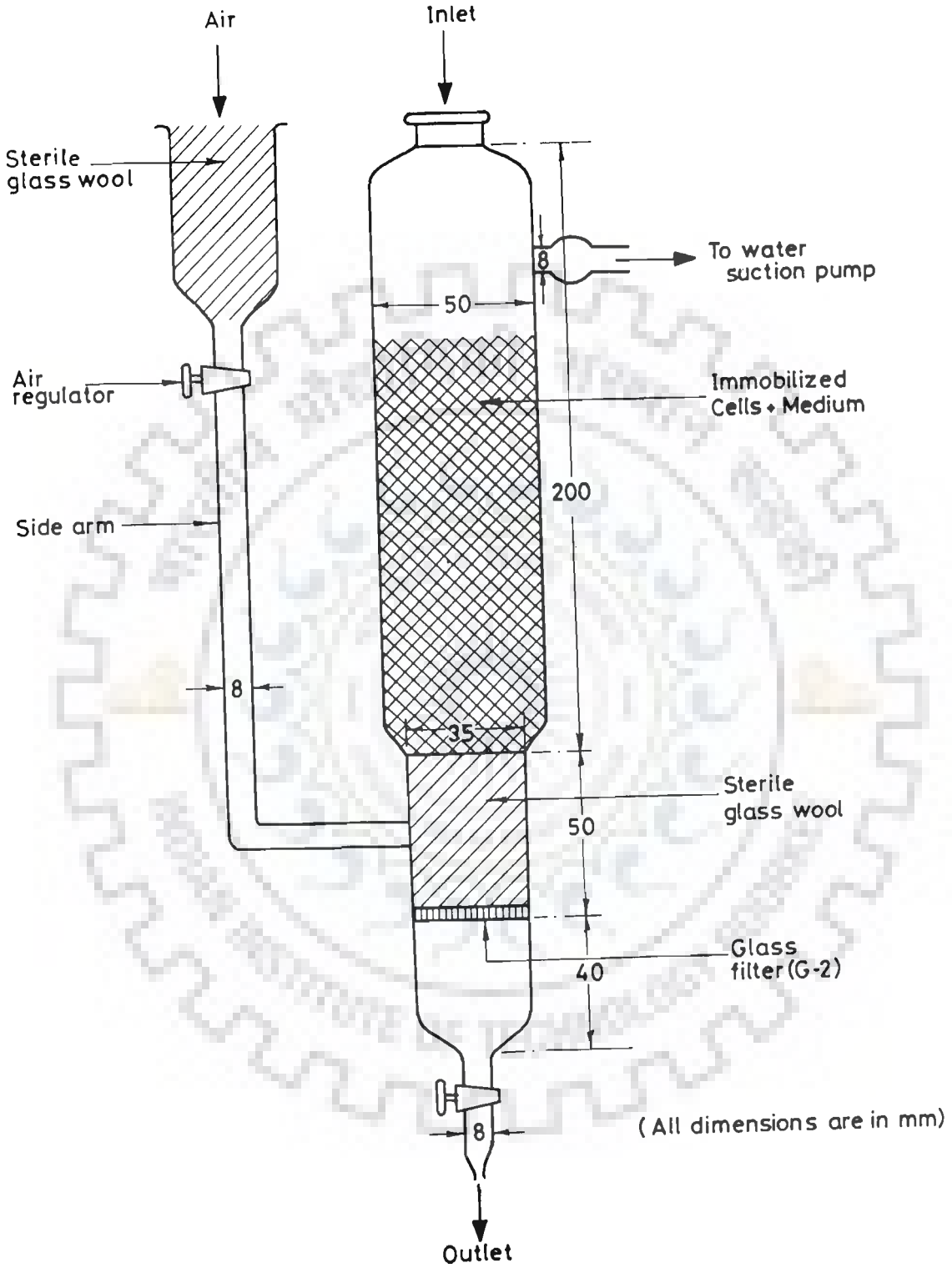

Air
Inlet
Sterile glass wool
To water suction pump
8
50
Air regulator
Immobilized Cells + Medium
200
Side arm
8
35
Sterile glass wool
50
Glass filter (G-2)
40
8
(All dimensions are in mm)
Outlet

Fig. 4

FIG.5 TWO-STAGE BIOREACTOR FOR IMMOBILIZED CELL FERMENTATION.

A two-stage bioreactor consisting of two single-stage bioreactors connected to each other in series. Each bioreactor is of 400 cm^3 (20x5 cm) with similar design as that of single stage bioreactor (Fig.4). After completion of fermentation in first stage, the fermented broth was fed into the second single-stage bioreactor for another fermentation cycle.

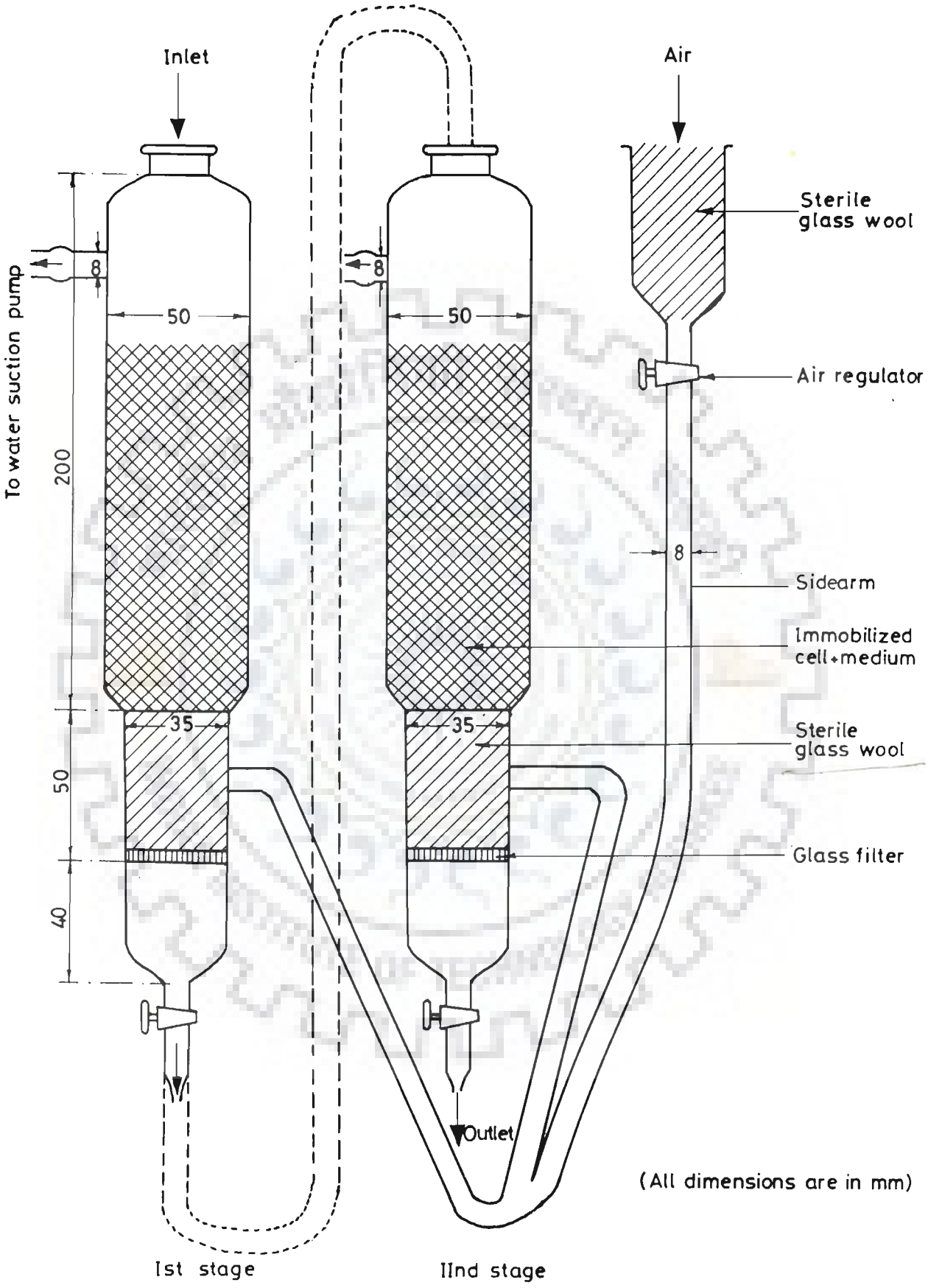

Inlet
Air
Sterile glass wool
Air regulator
To water suction pump
8
50
200
50
40
35
Sidearm
Immobilized cell+medium
Sterile glass wool
Glass filter
Outlet
(All dimensions are in mm)
Ist stage
IInd stage

Fig. 5

3.2.7 Analytical methods

The sugarcane molasses was suitably diluted with double distilled water and decolourized with charcoal. Total reducing sugars were determined with Fehling's reagent (ICUMSA, 1964). Sucrose content in the molasses was estimated by optical rotation method (ICUMSA, 1964). Sulphated ash by ICUMSA method (1964). Metals and phosphorus were assayed by atomic absorption spectroscopy. Protein was determined by Lowry's method (Lowry et al., 1951). Total titrable acidity was determined by titrating 1 ml sample aliquots (diluted with 5.0 ml of distilled water) with 0.05 N NaOH solution, using phenophthaline as an internal indicator. 1 ml of 0.05 N NaOH solution is equivalent to 3.413 mg/ml citric acid. Estimation of citric acid was done according to the method of Marrier and Boulet (1958).

For the determination of the dry weight of biomass, cells were separated from the fermentation broth by filtration through Whatman-41 filter paper, washed thoroughly with water, blotted and dried at 80°C for 24 h in an electric oven and weighed. The weight of the biomass was referred to as the dry weight of cells.

3.2.8 Product recovery

Citric acid was recovered by the classical calcium hydroxide precipitation method (Sodeck et al., 1981)(Fig.6).

3.2.9 Morphological study of fungus

For detailed morphological studies of fungus, Scanning Electron Microscopy (SEM) was carried out using Phillips 501 SEM. Pieces of fungal mat were taken and then the primary fixation was done, in 3% glutaraldehyde and 2% formaldehyde in 4:1 ratio, for 2 h (cf. Gabriel, 1982). After primary fixation samples were washed thrice with distilled water. These primary fixed samples were then passed through alcohol series (30%, 50%, 70%, 80%, 90% and 100%) for dehydration. Upto 70% alcohol, the samples were kept for 15 minutes, but afterwards the retention time was increased to 30 minutes. After passing

FIG.6 A FLOW DIAGRAMME SHOWING THE DOWN STREAM PROCEDURE FOR THE RECOVERY OF CITRIC ACID AFTER FERMENTATION.

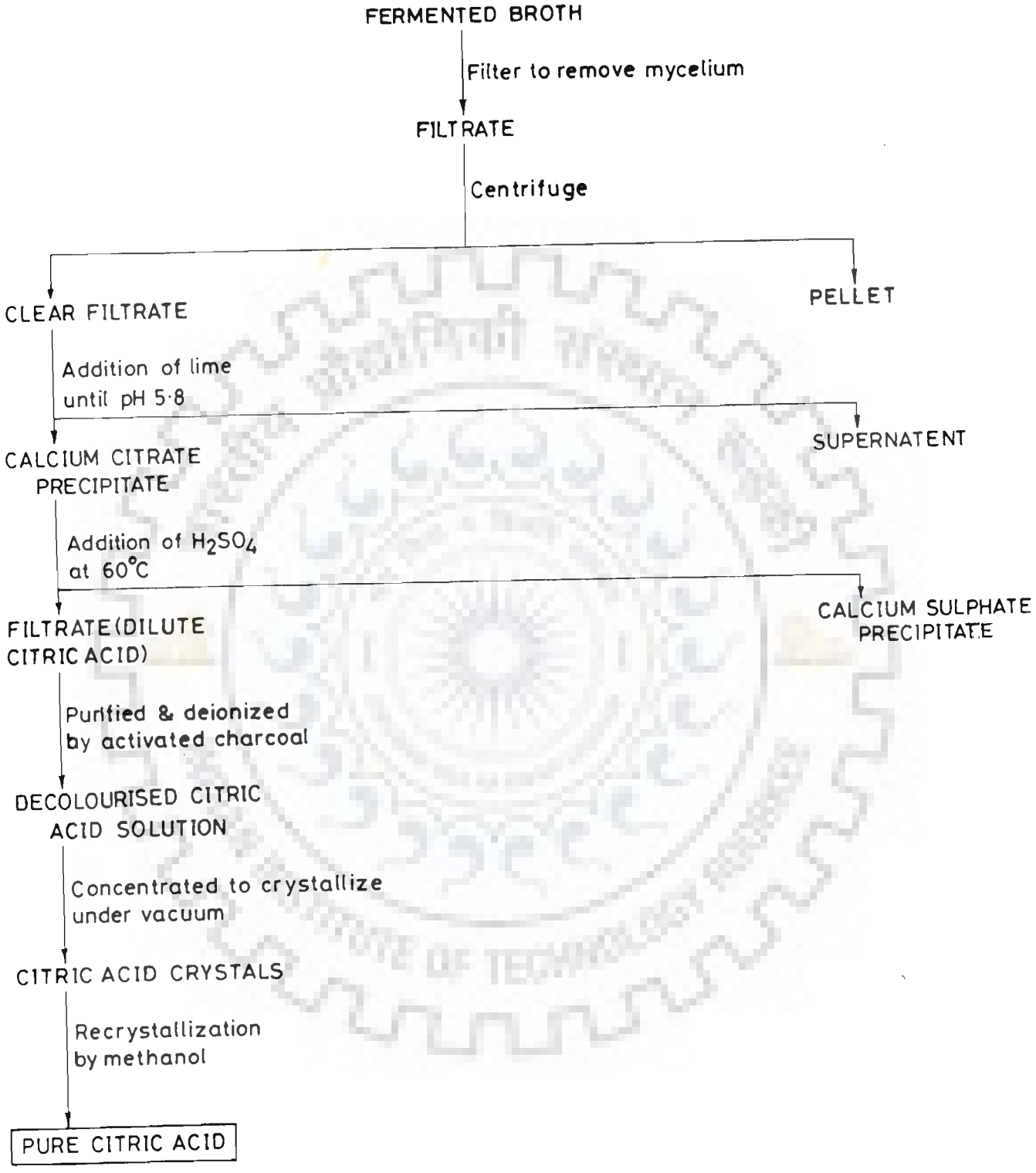

FERMENTED BROTH
Filter to remove mycelium
FILTRATE
Centrifuge
CLEAR FILTRATE
PELLET
Addition of lime until pH 5·8
SUPERNATENT
CALCIUM CITRATE PRECIPITATE
Addition of H_2SO_4 at 60°C
CALCIUM SULPHATE PRECIPITATE
FILTRATE (DILUTE CITRIC ACID)
Purified & deionized by activated charcoal
DECOLOURISED CITRIC ACID SOLUTION
Concentrated to crystallize under vacuum
CITRIC ACID CRYSTALS
Recrystallization by methanol
PURE CITRIC ACID

Fig. 6

through 100% alcohol, the samples were air dried (cf. Gabriel, 1982). These dried samples were examined under SEM by using gold-shadowing technique and electron photomicrographs were obtained at a required magnification.

4.0 RESULTS

4.1 IMPROVEMENT OF CITRIC ACID PRODUCING STRAIN OF *Aspergillus niger* BY UV MUTAGENESIS

For obtaining high citric acid yielding mutants of *Aspergillus niger,* the locally isolated wild type parental strain (*A. niger* KCB1) was subjected to UV mutagenesis. Precisely, wild type strain was grown on a potato-dextrose-agar (PDA) slant for 5 days at 30°C. Spores were suspended in sterilized water ($2x10^3$spores/ml) and single spores were spotted on a presterilized Whatman no.3 filter paper (120 mm diameter) soaked in a fermentation medium, pH 2.0, containing 10% of sucrose, 0.223% NH_4NO_3, 0.1% K_2HPO_4 and 0.023% $MgSO_4$ in a Petridish (120 x 17 mm), with the help of a capillary tube of 0.7 mm diameter. About 15 to 20 spores were singly planted per filter paper and were exposed to UV for different intervals varying from 10 min to 60 min. After mutagenic treatment the spores were incubated at 30°C for 115 h. A replica plate was prepared from the parent plate, spores were transferred to a PDA plate, grown for 5 days at 30°C and used for fermentation trials for citric acid production. After 115 h incubation, the parent plate was dried at 80°C and was subjected to a direct 'Acid Unitage test' by adding acetic anhydride and p-dimethylaminobenzaldehyde followed by heating at 140°C for 1 min. Purple coloured circular zones were produced around the spore colonies, indicating the formation of citric acid. The Acid Unit (AU) for each colony was determined by dividing the diameter of the circular purple zone around the spores by the diameter of the colony. A schematic representation of mutagenesis and screening is shown in Fig.7.

The results shown in Fig.8 clearly indicate a direct correlation between the AU values and the amount of citric acid produced during fermentation by different strains. Thus, use of AU values provided a simple and reliable method for screening the high citric acid yielding strains of *Aspergillus niger.*

On the basis of the AU values, distribution of various colonies after mutagenesis was determined. The results are shown schematically in Fig.9 and 10. Of the colonies that survived after 60 min UV exposure, it was found that about 81% of the cell population was in the 1.6-2.4 AU zone which corresponds to the parent strain. Approximately, 14% cell population was found to have an AU value of 1.0-1.6, indicating the minus mutants.

FIG.7 A DIAGRAMMATIC REPRESENTATION OF UV MUTAGENESIS AND FILTER PAPER CULTURE SCREENING METHOD.

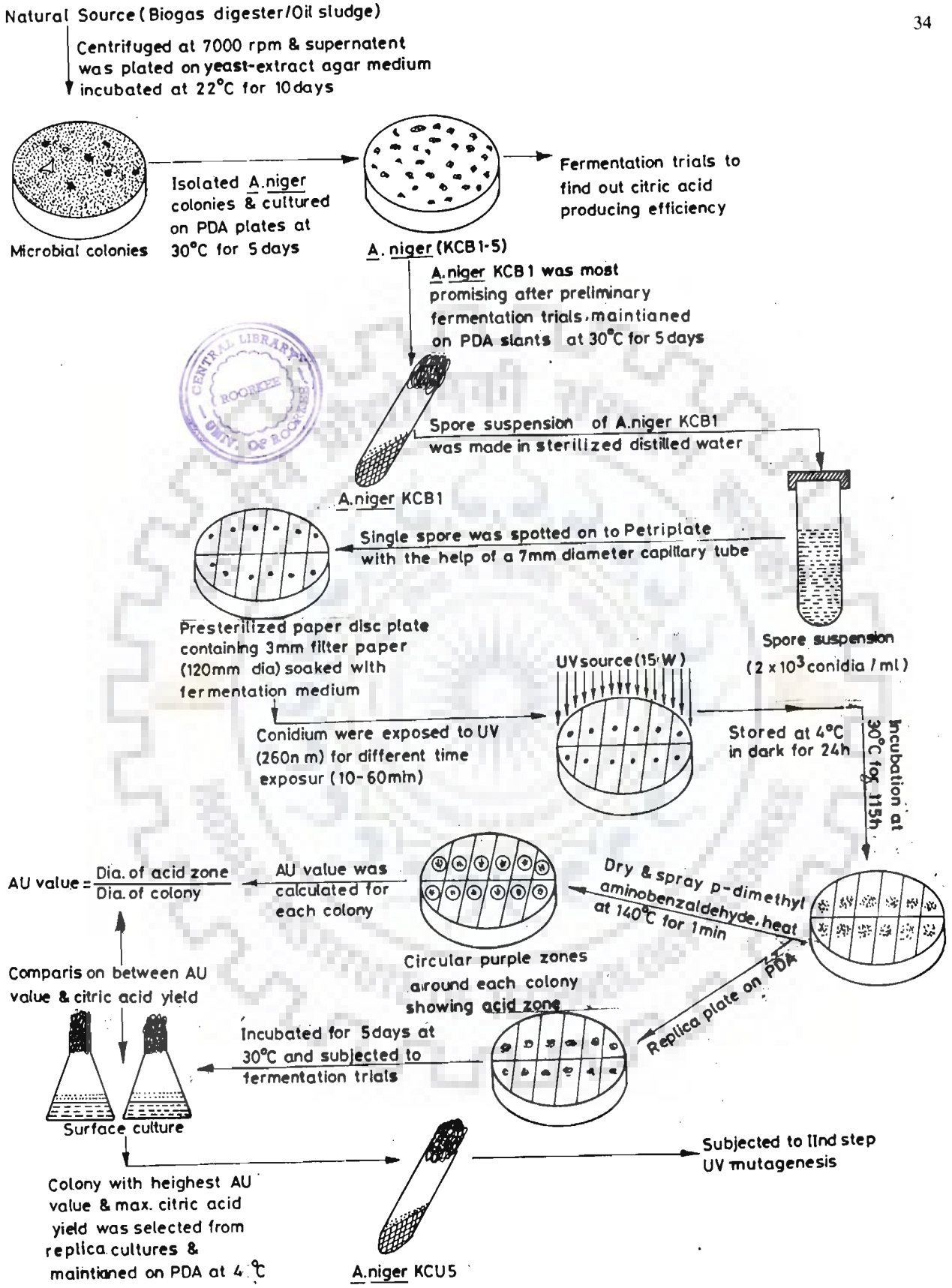

Natural Source (Biogas digester/Oil sludge)
Centrifuged at 7000 rpm & supernatent was plated on yeast-extract agar medium incubated at 22°C for 10 days
Microbial colonies
Isolated A.niger colonies & cultured on PDA plates at 30°C for 5 days
A. niger (KCB1-5)
Fermentation trials to find out citric acid producing efficiency
A.niger KCB1 was most promising after preliminary fermentation trials, maintianed on PDA slants at 30°C for 5 days
Spore suspension of A.niger KCB1 was made in sterilized distilled water
A.niger KCB1
Single spore was spotted on to Petriplate with the help of a 7mm diameter capillary tube
Presterilized paper disc plate containing 3mm filter paper (120mm dia) soaked with fermentation medium
Spore suspension (2 x 10³ conidia / ml)
UV source (15 W)
Conidium were exposed to UV (260n m) for different time exposur (10-60 min)
Stored at 4°C in dark for 24h
Incubation at 30°C for 115h
Dry & spray p-dimethyl aminobenzaldehyde, heat at 140°C for 1 min
Circular purple zones around each colony showing acid zone
AU value was calculated for each colony
AU value = Dia. of acid zone / Dia. of colony
Comparis on between AU value & citric acid yield
Replica plate on PDA
Incubated for 5days at 30°C and subjected to fermentation trials
Surface culture
Colony with heighest AU value & max. citric acid yield was selected from replica cultures & maintianed on PDA at 4 °C
A.niger KCU5
Subjected to IInd step UV mutagenesis

Fig. 7

FIG.8 A CORRELATION PLOT BETWEEN THE ACID UNITAGE (AU) VALUES AND AMOUNT OF CITRIC ACID PRODUCED.

AU values of various mutagenized colonies of *A. niger* were determined, as described under Methods, and their corresponding replica colonies were used to determine the citric acid yield by performing surface fermentation, using 10% sucrose as substrate in Doelger and Prescott's medium of pH 2.0, under well aerated conditions. Fermentation was carried out for 15 days at 30°C.

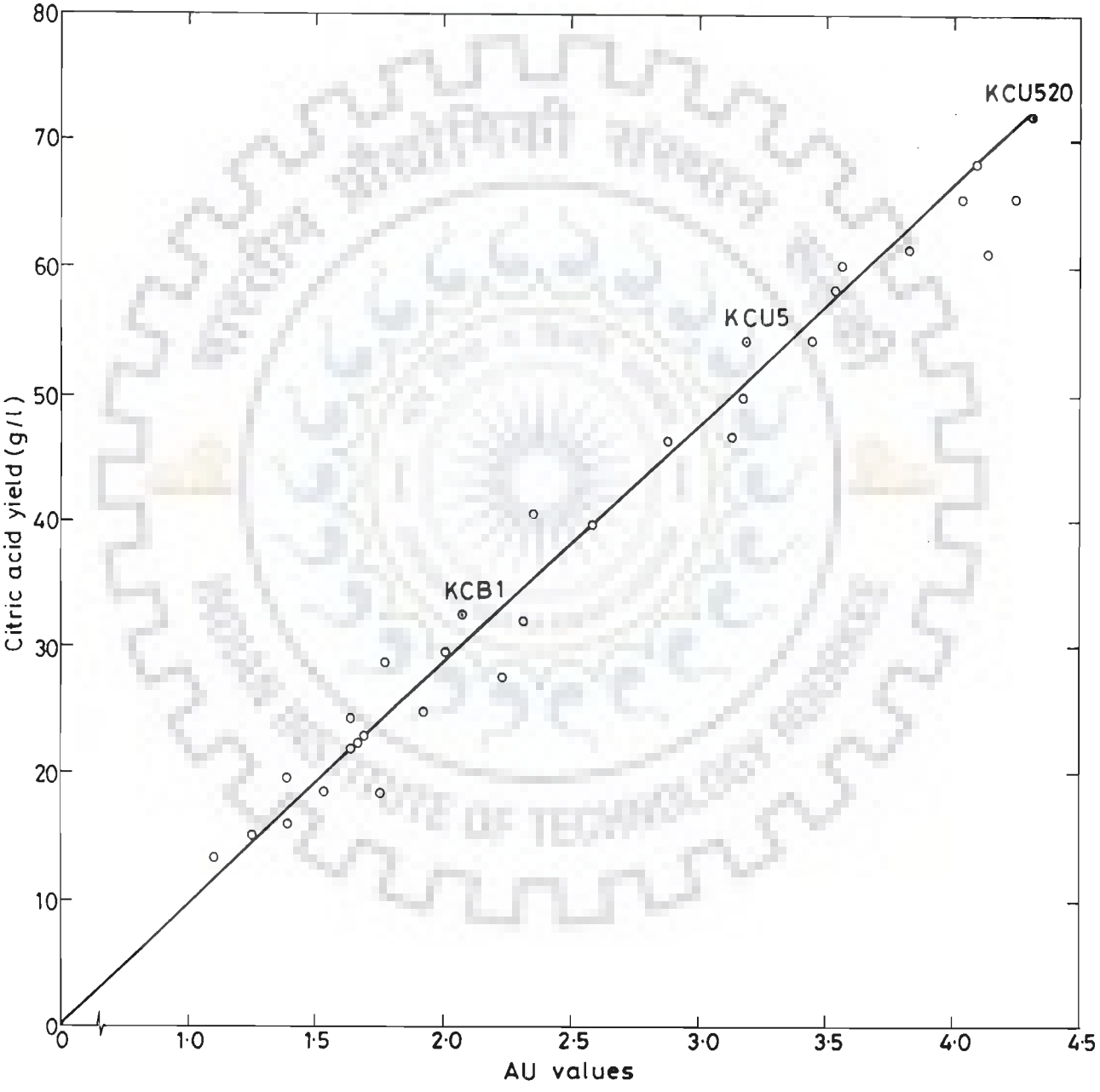

80
70
60
50
40
30
20
10
0
Citric acid yield (g/l)
KCU520
KCU5
KCB1
0
1·0
1·5
2·0
2·5
3·0
3·5
4·0
4·5
AU values

Fig. 8

FIG.9 Ist - STEP UV MUTAGENESIS OF A. niger.

The parental strain *A. niger* KCB1 was subjected to UV mutagenesis for different time intervals followed by screening of the survived colonies, as described under Methods. The percent distribution of the survived colonies to their corresponding AU values was calculated for each UV exposure. Various segments show percent distribution of colonies of *A. niger* in terms of AU values for citric acid producing ability.

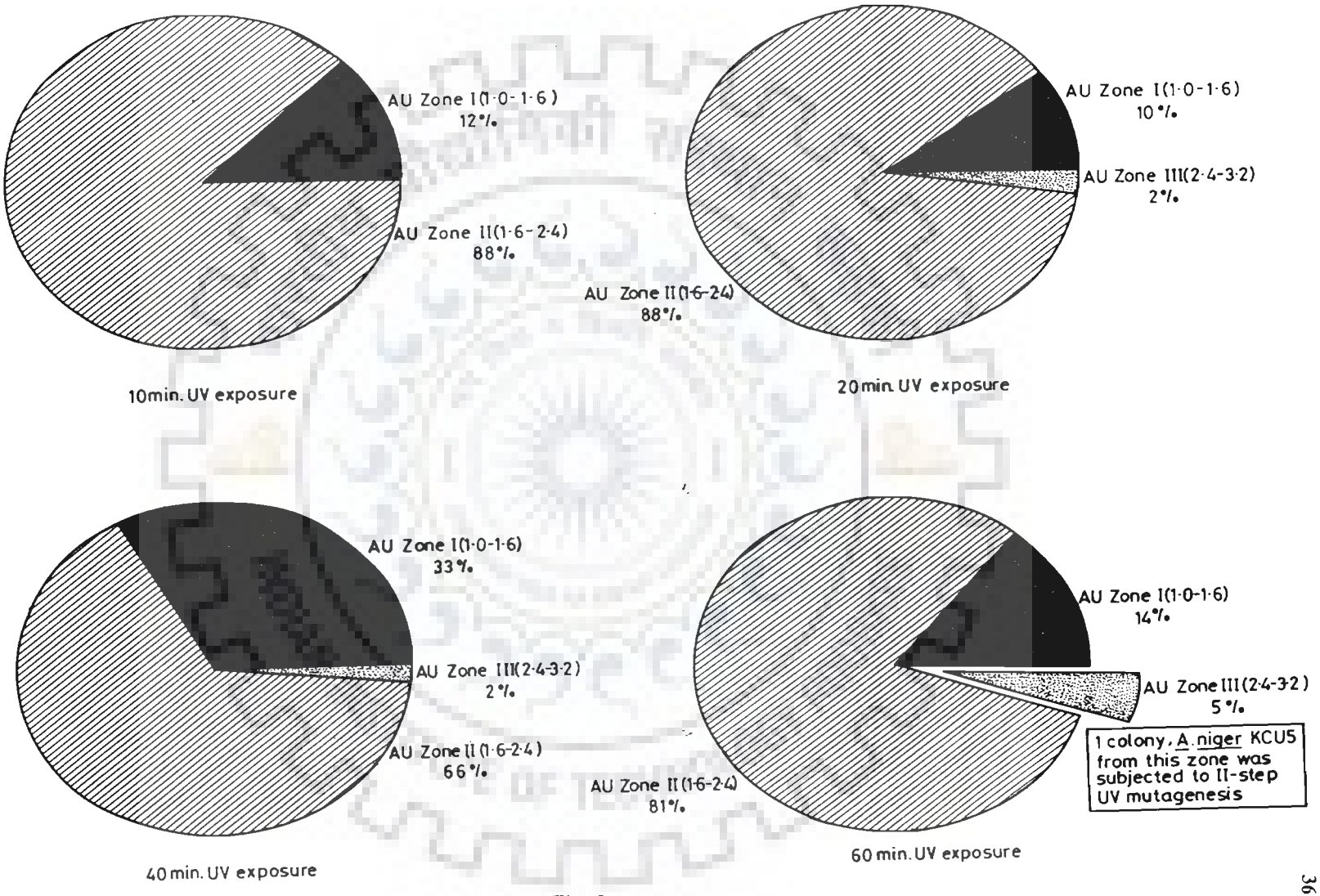

AU Zone I(1·0-1·6)
12%
AU Zone II(1·6-2·4)
88%
10min. UV exposure
AU Zone I(1·0-1·6)
10%
AU Zone III(2·4-3·2)
2%
AU Zone II(1·6-2·4)
88%
20min. UV exposure
AU Zone I(1·0-1·6)
33%
AU Zone III(2·4-3·2)
2%
AU Zone II(1·6-2·4)
66%
40 min. UV exposure
AU Zone I(1·0-1·6)
14%
AU Zone III(2·4-3·2)
5%
1 colony, A. niger KCU5 from this zone was subjected to II-step UV mutagenesis
AU Zone II(1·6-2·4)
81%
60 min. UV exposure

Fig. 9

FIG.10 IInd - STEP UV MUTAGENESIS OF A. niger.

The colony, showing highest AU value after Ist-step mutagenesis *(A. niger* KCU5), was isolated and subjected to IInd-step UV mutagenesis as described under Methods. After screening of the survived colonies, percent distribution of colonies according to their corresponding AU values was calculated. Various segments show the percent distribution of colonies of *A. niger* in terms of AU values for citric acid producing ability.

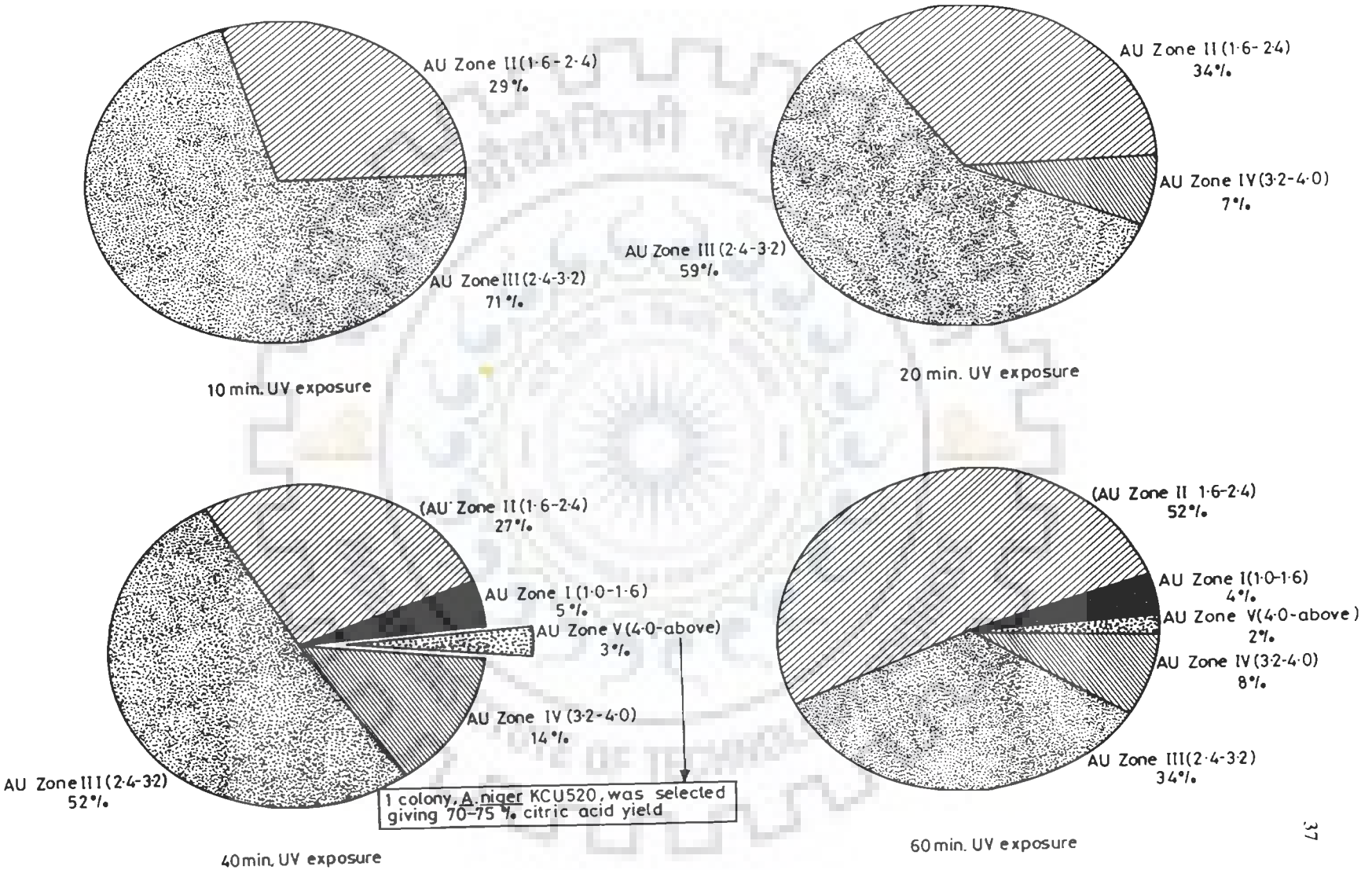

AU Zone II (1·6-2·4)
29 %
AU Zone III (2·4-3·2)
71 %
10 min. UV exposure
AU Zone III (2·4-3·2)
59%
AU Zone II (1·6-2·4)
34%
AU Zone IV (3·2-4·0)
7%
20 min. UV exposure
(AU Zone II (1·6-2·4)
27%
AU Zone I (1·0-1·6)
5%
AU Zone V (4·0-above)
3%
AU Zone IV (3·2-4·0)
14%
AU Zone III (2·4-3·2)
52%
40 min. UV exposure
1 colony, A. niger KCU520, was selected giving 70–75 % citric acid yield
(AU Zone II 1·6-2·4)
52%
AU Zone I (1·0-1·6)
4%
AU Zone V (4·0-above)
2%
AU Zone IV (3·2-4·0)
8%
AU Zone III (2·4-3·2)
34%
60 min. UV exposure

Fig. 10

However, about 5% cells were found to give AU value of 2.4-3.2. In other words 5 plus mutants were obtained in first step of UV mutagenesis.These were designated as *A. niger* KCU (1- 5). Of these mutants, *A. niger* KCU5 was found to be promising for citric acid fermentation with an AU value of 3.2 (Fig.9).

The mutant *A. niger* KCU5 was then subjected to second-step UV mutagenesis. Following the standard screening procedure based on AU values, as described, as many as 20 plus mutants (*A. niger* KCU5, 1-20) were obtained. The overall distribution of cell population at various intervals of UV exposure is shown in Fig.10. It was interesting to find that out of the 200 colonies that were screened for higher citric acid yields, 3% colonies were found to have AU of 4.0 or above. Of these *A. niger* KCU520 was found to convert 70-75% carbohydrate into citric acid, which was nearly 2x more efficient than the parent strain *A. niger* KCB1. This strain was maintained on a PDA plate, stored at 4^{0}C and used in the present study.

4.1.1 Morphology of mutant *A. niger* KCU520

In the course of strain selection it was observed that the parent strain *A. niger* KCB1 was light yellow in colour, whereas after first step UV mutagenesis, in addition to the normal yellow coloured colonies, 5 colonies, designated as *A. niger* KCU1-5, of apparently white colour were obtained. Interestingly, these colonies also showed higher AU values than the parent strain. In fact, it provided us a working handle for the primary screening of high citric acid producing *Aspergillus niger* strains. The final strain *A. niger* KCU520 was also white in colour (Fig.11).

The morphology of the mutant *A. niger* KCU520 and the parent strain *A. niger* KCB1 were also investigated by scanning electron microscopy. Fig.12 shows the electron micrographs of the parent and mutant strains taken under same magnification. It is clearly evident from these electron micrographs that morphologically the mutant *A. niger* KCU520 is distinctly different from the parent strain *A. niger* KCB1. For example, the parent strain was thin and a thread-like elongated structure, while the mutant *A. niger* KCU520 was thick, short and swollen with rough cell surface. In the latter, some branches were also apparent which were not visible in the parent strain even at higher magnification (Fig.12). To what extent

FIG.11 MORPHOLOGICAL DIFFERENCE BETWEEN THE PARENT AND THE MUTANT STRAIN OF A. niger.

* Top panel (left) shows a yellow coloured mycelium of parent *A. niger* KCB1 grown during the surface fermentation on a 10% sucrose medium, and (right) a white coloured mycelium of mutant culture *A. niger* KCU520. The photographs of the mycelium were taken from the reverse side, i.e., the side which was in contact with the medium.

* Bottom panel (left) shows a thick spore population on the mycelium of *A. niger* KCB1 grown during surface fermentation, and (right) a thin population of spores of the *A. niger* KCU520 mycelium under identical fermentation conditions. The photographs of mycelium were taken from the upper side of the culture.

Fig.11

FIG.12 SCANNING ELECTRON MICROGRAPHS OF KCB1 AND KCU520 STRAINS OF A. niger.

Detailed morphology of *A. niger* KCB1 and *A. niger* KCU520 is compared. After surface citric acid fermentation with KCB1 and KCU520 strains of *A. niger,* the fungal mycelium mat was separated, thoroughly washed, blotted and subjected to primary fixation followed by dehydration as described under Methods. The air dried fungal pieces were examined under SEM (PSEM 501) using gold-shadowing technique.

(a) **UPPER PANEL- KCB1 (left) and KCU520 (right) 320 x.**

(b) **MIDDLE PANEL- KCB1 (left) and KCU520 (right) 640 x.**

(c) **LOWER PANEL- KCB1 (left) and KCU520 (right) 1250 x.**

Note the short, swollen and branched structure of the mutant cell compared to the thin, smooth and thread like structure of the wild type.

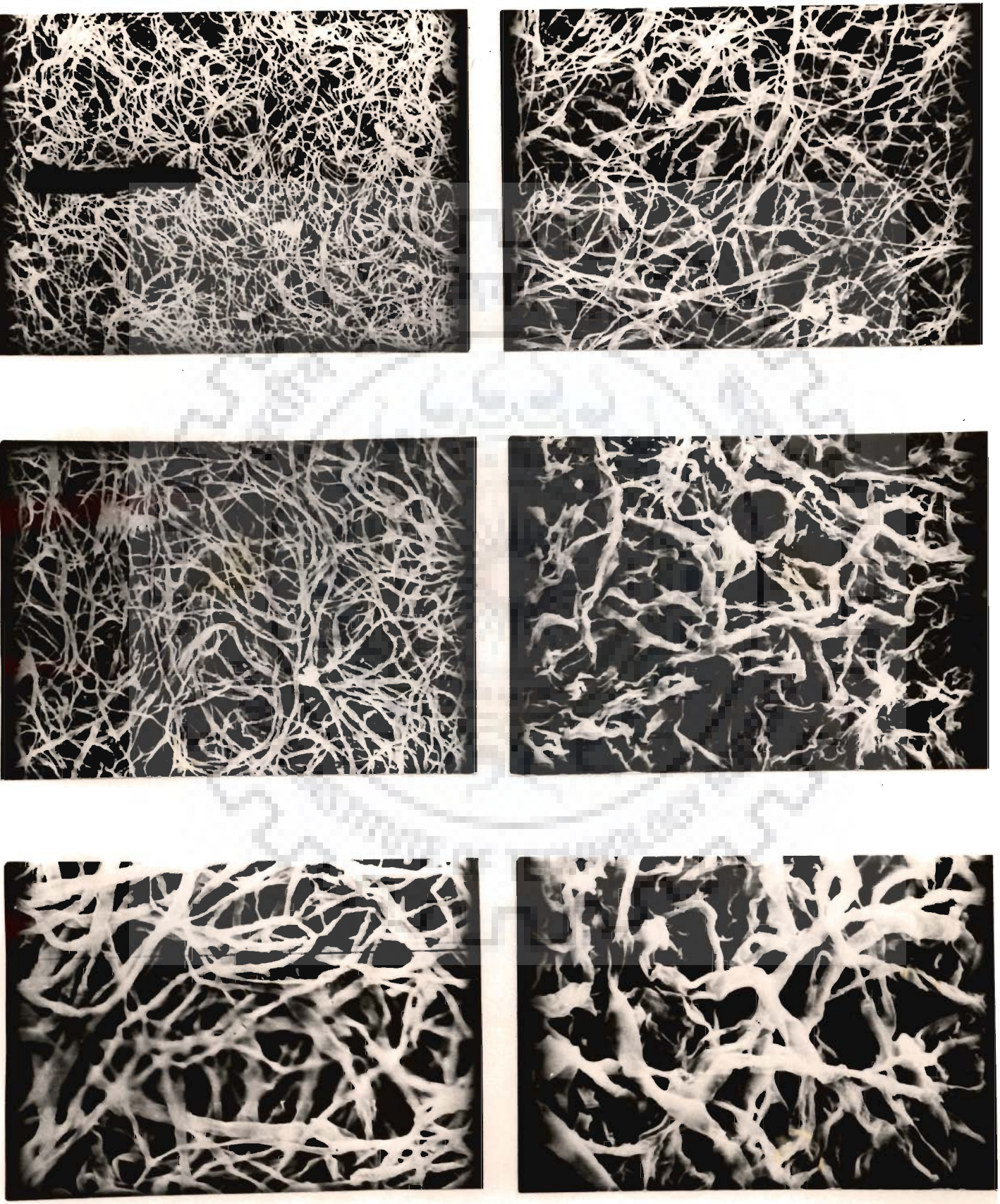


Fig.12

the altered morphology of the strain *A. niger* KCU520 is responsible for higher citric acid production from carbohydrates is not clear at the moment.

4.2 OPTIMIZATION OF CITRIC ACID FERMENTATION

The conditions for citric acid formation by surface fermentation were optimized using high citric acid yielding strain *A. niger* KCU520, and sucrose as substrate. Unless stated otherwise, surface fermentation was carried out at 30°C for 15 days. Both biomass and citric acid yields were estimated. For the sake of comparison, fermentation was also performed in parallel experiments under identical conditions using wild type strain *A. niger* KCB1. Results of various experiments are given below:

4.2.1 Citric acid fermentation as a function of sugar source

Effect of carbon source on the citric acid production is shown in Fig.13. It was clearly observed that out of different sugar sources used, sucrose was found to be the best, followed by glucose, glycerol and fructose. Galactose was found to be unfit for citric acid production.

4.2.2 Citric acid fermentation as a function of substrate concentration

The effect of substrate (sucrose) concentration on the production of citric acid and biomass after 15 days surface fermentation at 30°C is shown in Fig.14. It can be seen that the rates of production of citric acid and biomass in the case of mutant *A. niger* KCU520 are clearly different from that of the parent strain, *A. niger* KCB1. In the case of *A. niger* KCU520, maximum citric acid yield (75% conversion of carbohydrates to citric acid) was attained when the sucrose concentration was between 12-14%. Beyond 14% there was a sudden decline in the citric acid production, but in contrast, the biomass was unchanged showing only marginal increase. It was also observed that at optimum substrate concentration (14% sucrose) citric acid to biomass ratios for *A. niger* KCU520 and *A. niger* KCB1 were approximately 10 and 2, respectively. These results further support the superiority of

FIG.13 CITRIC ACID FERMENTATION AS A FUNCTION OF SUGAR SOURCE.

Surface citric acid fermentation was carried out with *A. niger* KCU520 using various sugar source at a concentration of 10% in a Doelger and Prescott medium of pH 2.0 under well aerated conditions (2 l/min). After 15 days of fermentation at 30°C, citric acid (□) and dry cell mass (▨) were determined as described under Methods. S, Gu, F, L, Ga, M and Gy represent sucrose, glucose, fructose, lactose, galactose, maltose and glycerol, carbon sources used for fermentation respectively.

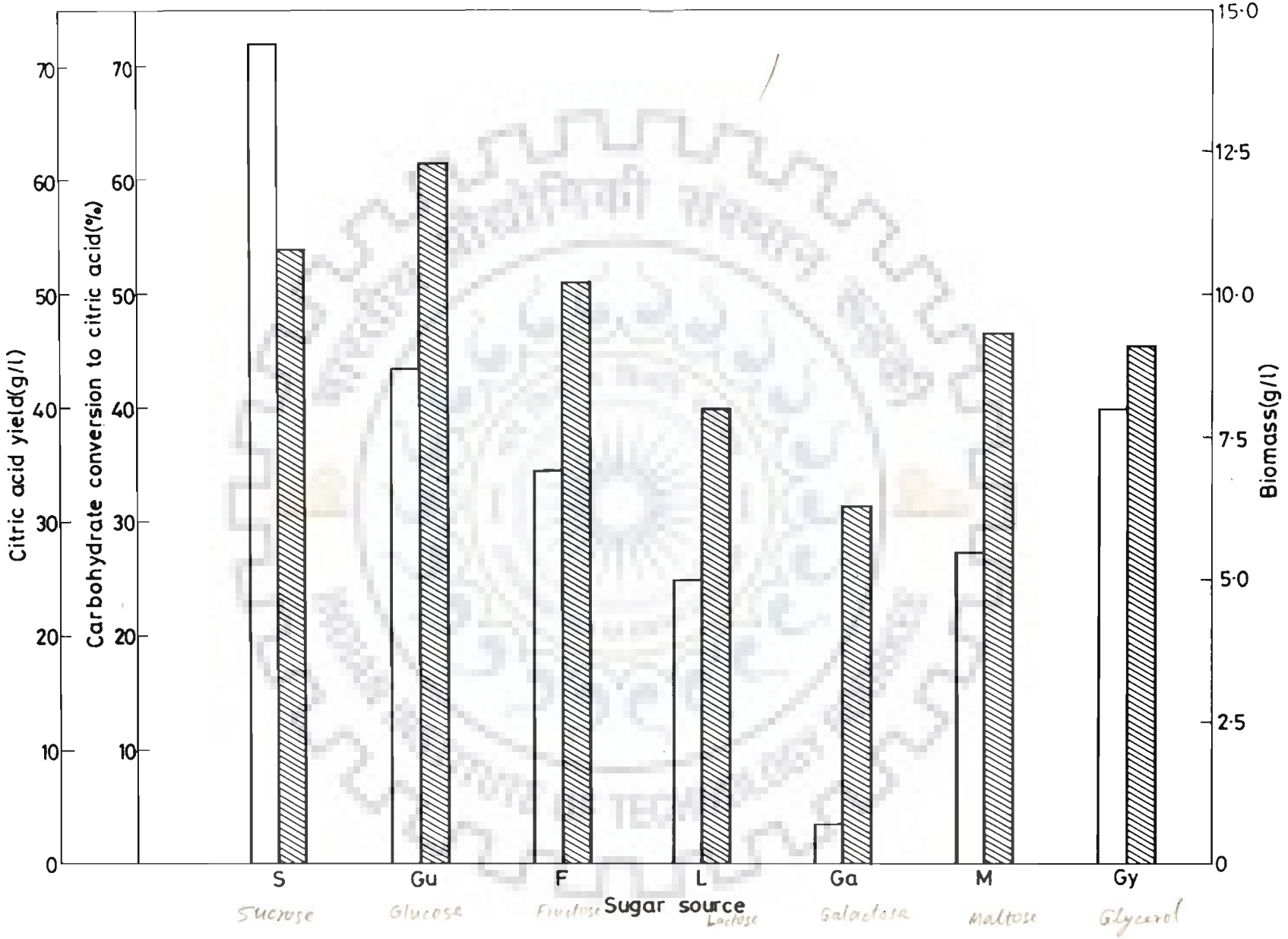

Citric acid yield(g/l)
Carbohydrate conversion to citric acid(%)
Biomass(g/l)
0
10
20
30
40
50
60
70
2·5
5·0
7·5
10·0
12·5
15·0
S
Gu
F
L
Ga
M
Gy
Sucrose
Glucose
Fructose
Lactose
Galactose
Maltose
Glycerol
Sugar source

Fig. 13

FIG.14 CITRIC ACID FERMENTATION AS A FUNCTION OF SUBSTRATE CONCENTRATION.

Surface citric acid fermentation was carried out with KCU520 and KCB1 strains of *A. niger* using varying concentration of sucrose in Doelger and Prescott medium of pH 2.0 under well aerated conditions (2 l/min). After 15 days of fermentation at 30°C, citric acid and dry biomass were determined according to Methods. (○—○), (●—●) shows citric acid and (Δ--Δ), (▲--▲) shows biomass produced by *A. niger* KCU520 and *A. niger* KCB1, respectively.

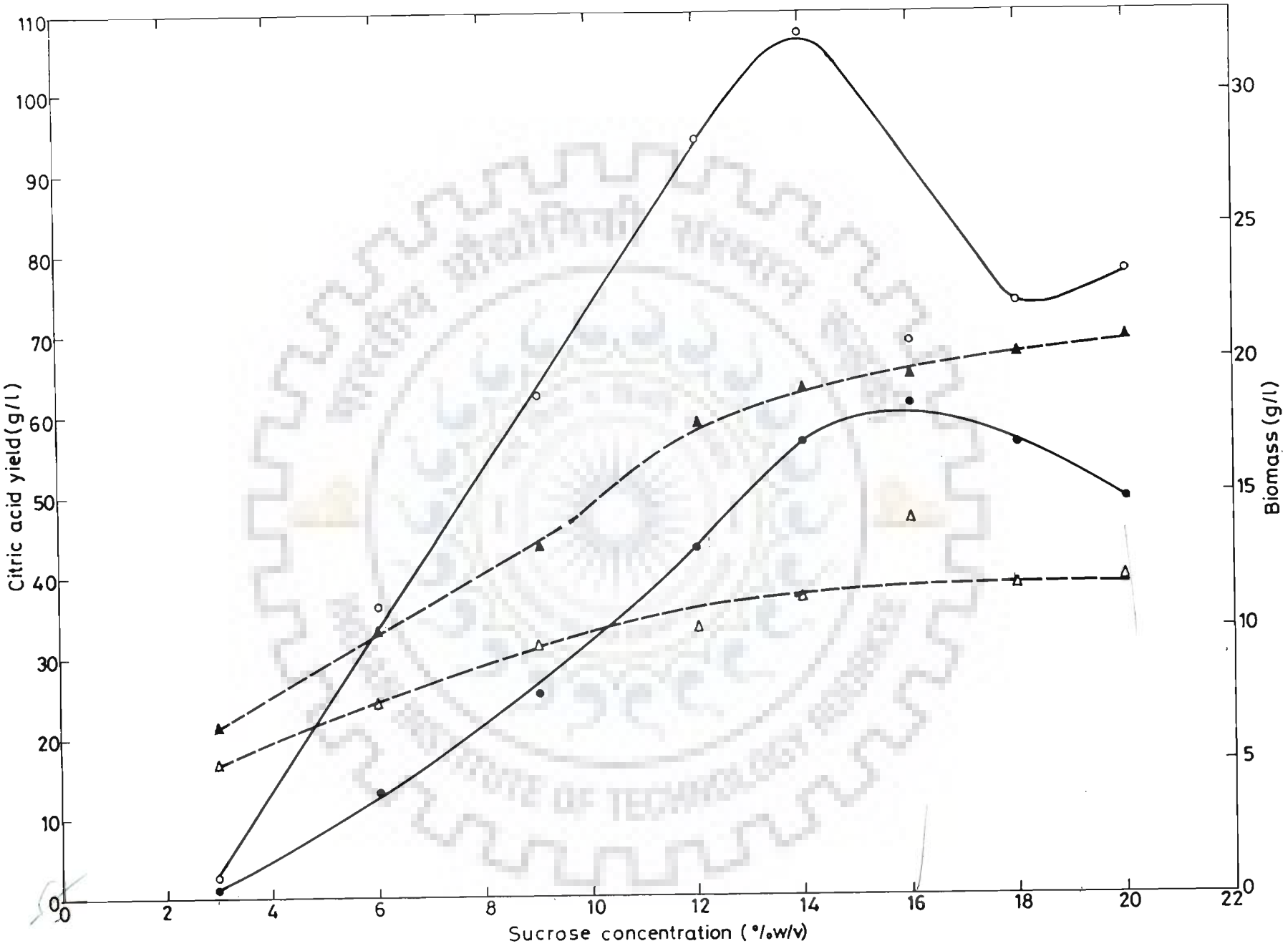

Citric acid yield (g/l)
Biomass (g/l)
Sucrose concentration (°/o w/v)
0
10
20
30
40
50
60
70
80
90
100
110
0
5
10
15
20
25
30
0
2
4
6
8
10
12
14
16
18
20
22

Fig. 14

KCU520 over KCB1 strain of *A. niger* and that in case of *A. niger* KCU520 more substrate was converted to citric acid and less was used for cell mass production. In contrast, the rate of citric acid formation by *A. niger* KCB1 was much slower than that of *A. niger* KCU520, but the former's cell mass was greater than the latter. On the basis of these results 12-14% sucrose solution was used in all other experiments described below.

4.2.3 Citric acid fermentation as a function of spore age

Fig.15 shows the effect of spore age on formation of citric acid and biomass. The results indicate that 5-day old spores of *A. niger* KCU520 were best suited for citric acid fermentation, giving nearly 75% carbohydrate conversion to citric acid. *A. niger* KCB1, on the other hand, exhibited a wider spore age range (3-5 days) for maximum citric acid production (Fig.15). The biomass pattern for both strains was nearly similar except that *A. niger* KCB1 showed more fungal growth compared to *A. niger* KCU520. In both cases, biomass gradually increased upto 9th day and thereafter dropped rapidly showing a degeneration phase.

4.2.4 Citric acid fermentation as a function of temperature

match the order with fig. 16.

The optimum temperature for citric acid formation by KCU520 and KCB1 strains of *A. niger* was found to be between 26-28°C and 28-30°C, respectively (Fig.16). The biomass shows a gradual increase with increase in temperature in both cases. Again, as expected, parental strain *A. niger* KCB1 shows more growth as compared to *A. niger* KCU520, under similar fermentation conditions.

4.2.5 Citric acid fermentation as a function of pH

Fig.17 shows the citric acid and biomass profile with respect to pH for KCU520 and KCB1 strains of *A. niger*. In both cases, two pH optima (pH 1.75 and 4.0) were observed, with pH 1.75 being more predominant. Occurrence of two pH optima seems to be an inherent property of *A. niger* KCB1 which is sustained in the mutant. Thus, the results show a possibility of producing citric acid at pH 4.0. Nevertheless, it is clear that at pH 1.75 the

FIG.15 CITRIC ACID FERMENTATION AS A FUNCTION OF AGE OF SPORES.

A. niger KCB1 and *A. niger* KCU520 were subcultured onto potato dextrose agar (PDA) Petriplates and incubated at 30°C for an indicated period. Spores obtained after different time intervals were used to inoculate the Doelger and Prescott medium of pH 2.0. After 15 days of surface fermentation at 30°C under well aerated conditions (2 l/min), citric acid and dry biomass were measured as described under Methods. (o—o), (•—•) shows citric acid and (Δ--Δ), (▲--▲) shows biomass produced by *A. niger* KCU520 and *A. niger* KCB1, respectively.

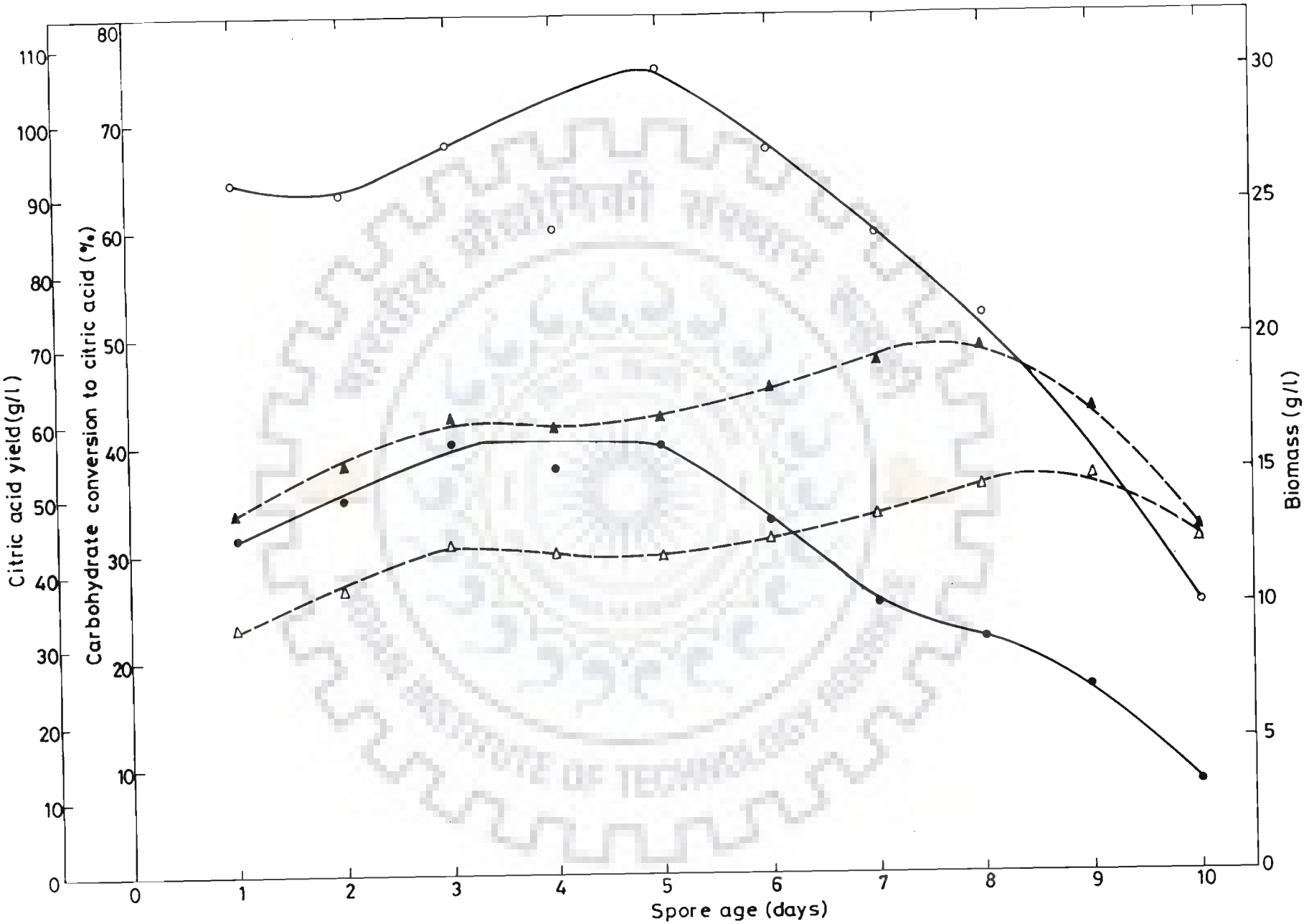

Citric acid yield (g/l)
Carbohydrate conversion to citric acid (%)
Biomass (g/l)
Spore age (days)
0
1
2
3
4
5
6
7
8
9
10
110
100
90
80
70
60
50
40
30
20
10
0
80
70
60
50
40
30
20
10
0
30
25
20
15
10
5
0

Fig. 15

FIG.16 CITRIC ACID FERMENTATION AS A FUNCTION OF TEMPERATURE.

Surface citric acid fermentation was carried out with KCB1 and KCU520 strains of *A. niger* by employing Doelger and Prescott medium of pH 2.0. Fermentation was carried out under well aerated conditions at varying temperatures for 15 days, followed by determination of citric acid and dry cell mass as described under Methods. (o——o), (●——●) shows citric acid and (Δ- -Δ), (▲--▲) shows biomass produced by *A. niger* KCU520 and *A. niger* KCB1, respectively.

dry as at other places

Strain no. must be in the same order at all the places

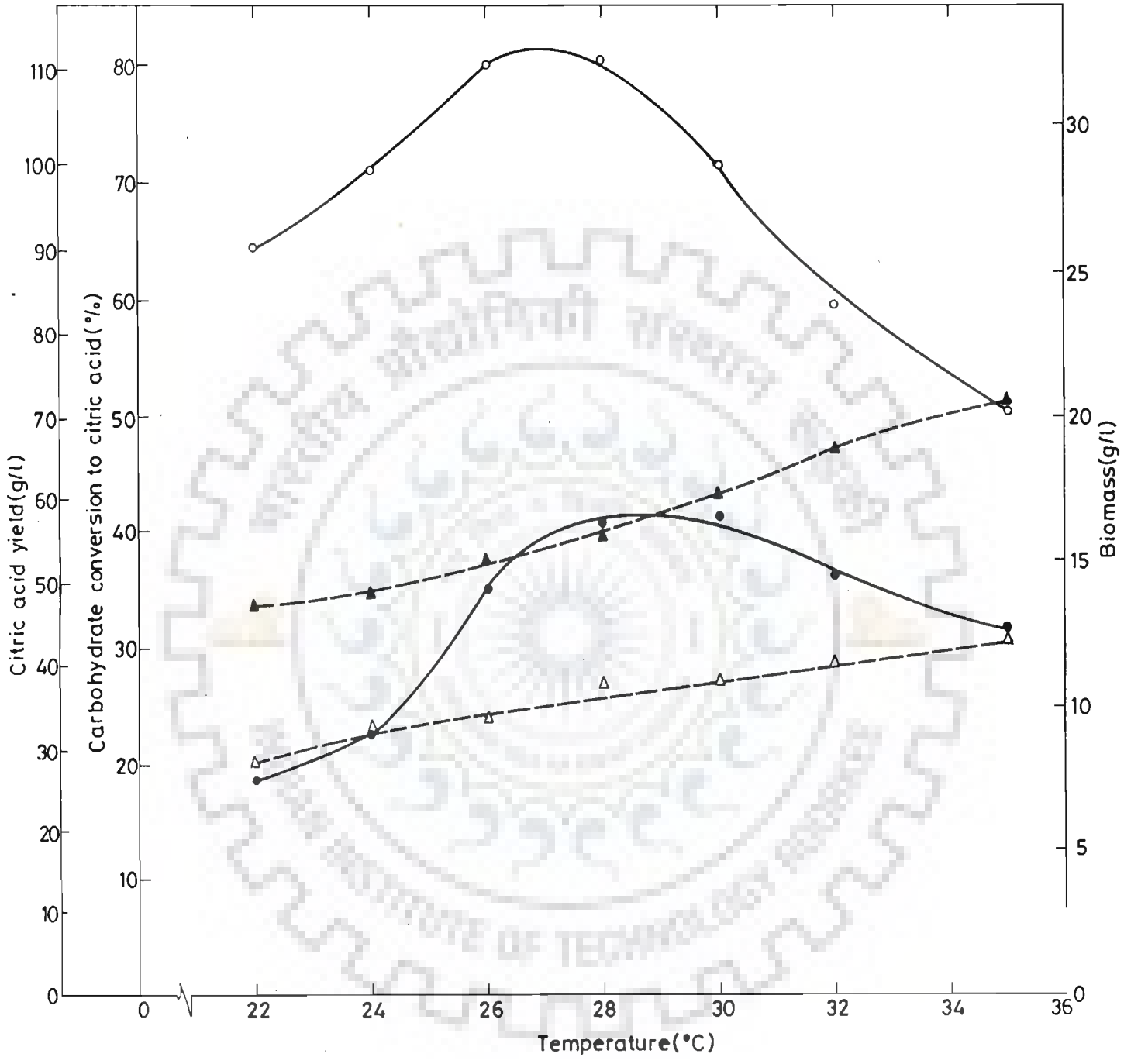

Citric acid yield(g/l)
Carbohydrate conversion to citric acid(%)
Biomass(g/l)
Temperature(°C)
0
22
24
26
28
30
32
34
36
10
20
30
40
50
60
70
80
90
100
110
5
15
25

Fig. 16

FIG.17 CITRIC ACID FERMENTATION AS A FUNCTION OF pH.

Surface citric acid fermentation was carried out with *A. niger* KCB1 and *A. niger* KCU520 in Doelger and Prescott medium, adjusted to the desired initial pH by 1 N HCl. After 15 days of fermentation at 30°C under high aeration rates (2 l/min), citric acid and dry biomass were measured as described under Methods. (o——o), (●——●) shows citric acid and (Δ- - -Δ), (▲- - -▲) shows biomass produced by *A. niger* KCU520 and *A. niger* KCB1, respectively.

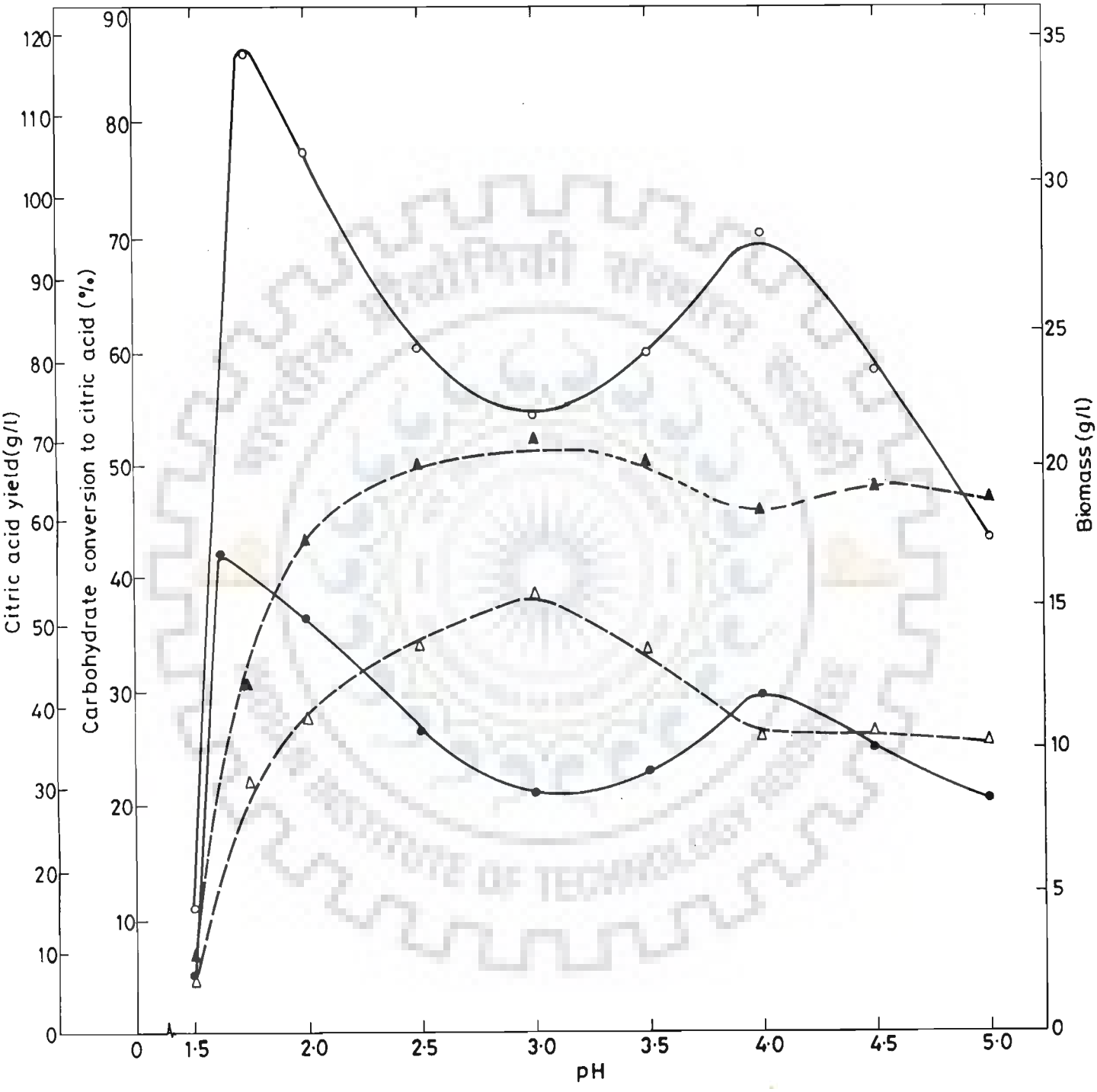

Citric acid yield(g/l)
Carbohydrate conversion to citric acid (%)
Biomass (g/l)
pH
0
1·5
2·0
2·5
3·0
3·5
4·0
4·5
5·0

Fig. 17

conversion of sucrose to citric acid was about 85% compared to about 77% at pH 4.0. Thus, in all experiments fermentation was carried out at pH 1.75. Also, at this pH, chances of microbial contamination were reduced drastically.

4.2.6 Citric acid fermentation as a function of inoculum level

Results in Fig.18 show that citric acid formation is independent of inoculum level. For instance, even at 1% level of inoculum the amount of citric acid formed by *A. niger* KCU520 is comparable with the amount formed at 8-10% inoculum level. Hence, we have used 1-2% inoculum level in fermentation experiments.

4.2.7 Citric acid fermentation as a function of fermentation time

Fig.19 shows the time dependence of citric acid fermentation by KCU520 and KCB1 strains of *A. niger.* It was found that *A. niger* KCU520 took nearly 10-12 days for the completion of fermentation which is significantly shorter than required by *A. niger* KCB1. When these results were transformed on a per day basis, a clear picture of citric acid fermentation emerged (Fig.20). In the course of fermentation there are two distinct growth phases and production phases. It was observed that each citric acid production phase is preceded by a growth phase.

4.2.8 Citric acid fermentation as a function of medium composition

In order to increase the rate of citric acid formation, effect of major nutrients on the citric acid fermentation was investigated. It was observed that maximum amount of citric acid accumulation occurred when 0.25% NH_4NO_3; 0.1% K_2HPO_4 and 0.03% $MgSO_4$ were present in the medium (Table-1). The citric acid yield was also greatly influenced by the N:P ratio.

FIG.18 CITRIC ACID FERMENTATION AS A FUNCTION OF INOCULUM LEVEL.

Surface citric acid fermentation was carried out with KCB1 and KCU520 strains of *A. niger* in Doelger and Prescott medium. Medium was inoculated with different amounts of inoculum containing 10^6-10^7 spores/ml. After 15 days of fermentation under well aerated conditions, citric acid and dry biomass were determined according to Methods. (○—○), (●—●) shows citric acid and (△--△), (▲--▲) shows biomass produced by *A. niger* KCU520 and *A. niger* KCB1, respectively.

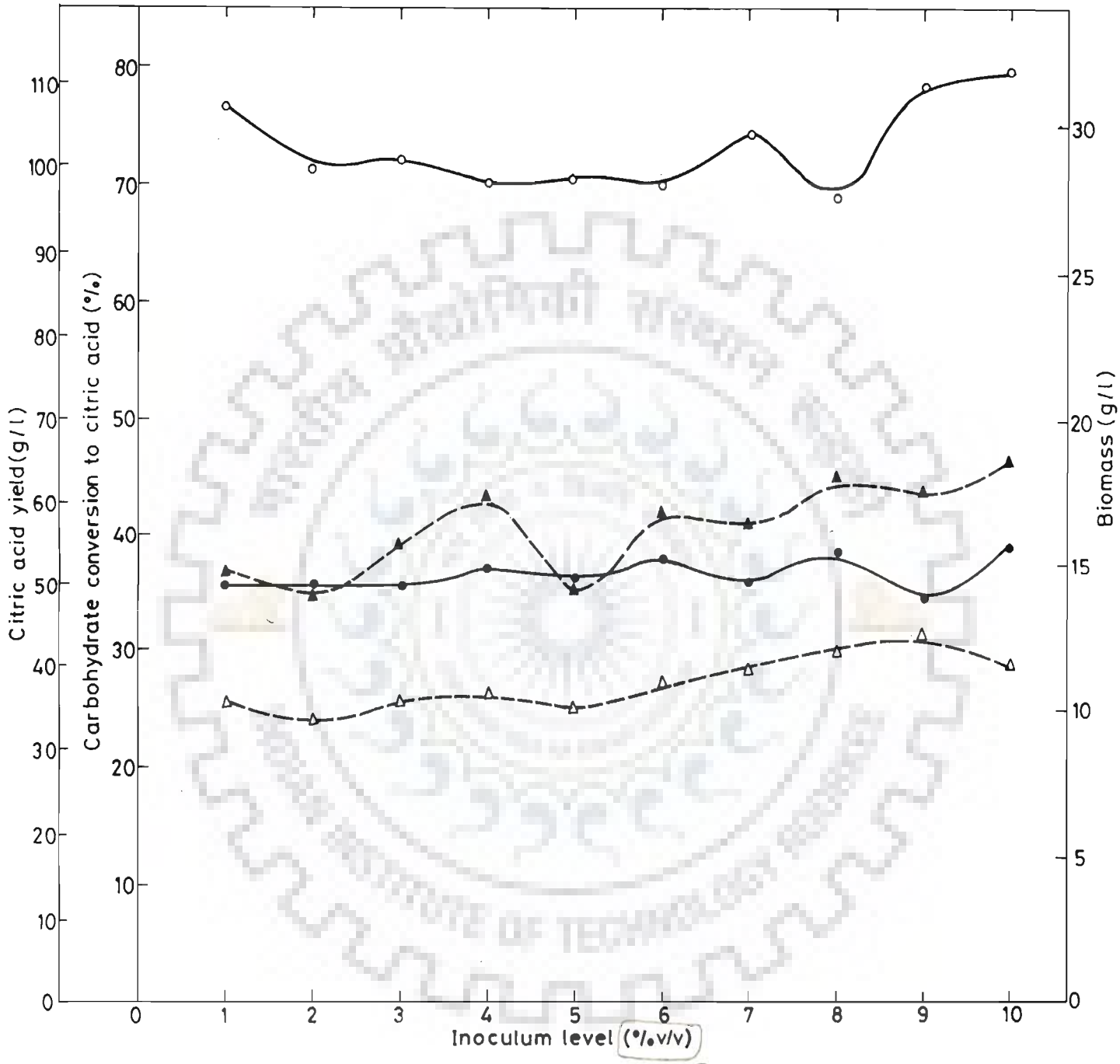

Citric acid yield(g/l)
Carbohydrate conversion to citric acid (%)
Biomass (g/l)
Inoculum level (%v/v)
0
1
2
3
4
5
6
7
8
9
10
10
20
30
40
50
60
70
80
90
100
110
0
5
10
15
20
25
30

Fig. 18

FIG.19 CITRIC ACID FERMENTATION AS A FUNCTION OF FERMENTATION TIME.

Surface citric acid fermentation was carried out with KCB1 and KCU520 strains of *A. niger* by employing Doelger and Prescott medium under optimum fermentation conditions. Fermentation was terminated after the indicated periods and citric acid and dry biomass were determined as described under Methods. (o—o), (●—●) shows citric acid and (Δ--Δ), (▲--▲) shows biomass produced by *A. niger* KCU520 and *A. niger* KCB1, respectively.

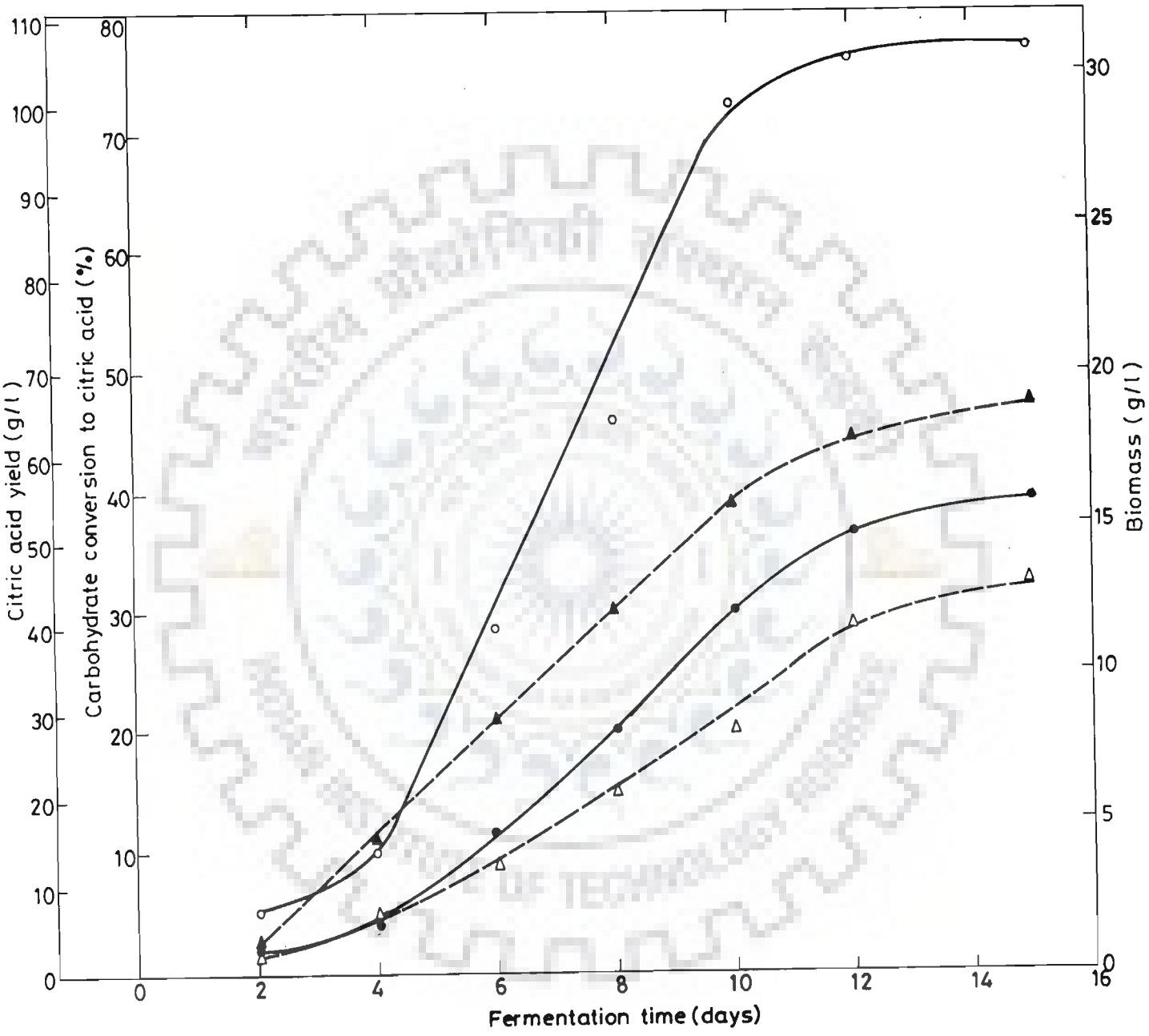


Fig. 19

FIG.20 RATE OF CITRIC ACID AND BIOMASS PRODUCTION DURING FERMENTATION.

The rate of citric acid and biomass production at 2,4,6,8,10,12 and 15 days of fermentation was computed as follows :

Total amount of citric acid or biomass in 'n'days of fermentation - Total amount of citric acid or biomass in 'n-2' days of fermentation / 2.

The results are plotted as rate of citric acid or biomass/day Vs. total days of fermentation. (o——o), (●——●) shows citric acid and (Δ- - -Δ), (▲- - -▲) shows biomass production rate of *A. niger* KCU520 and *A. niger* KCB1, respectively.

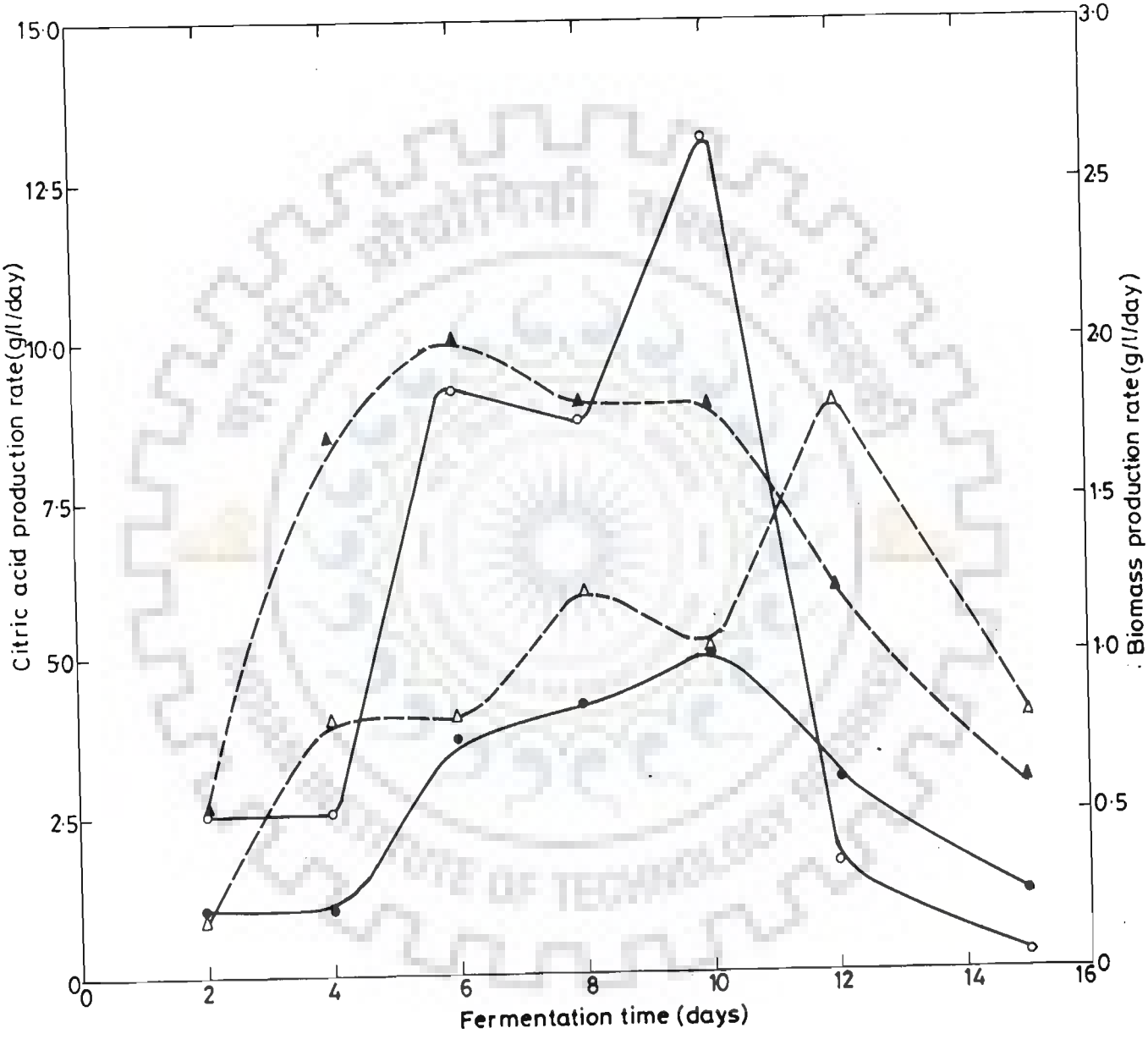

15·0
12·5
10·0
7·5
5·0
2·5
0
Citric acid production rate(g/l/day)
3·0
2·5
2·0
1·5
1·0
0·5
0
Biomass production rate(g/l/day)
0
2
4
6
8
10
12
14
16
Fermentation time (days)

Fig. 20

TABLE-1 EFFECT OF MAJOR NUTRIENTS ON THE CITRIC ACID FERMENTATION.

Surface citric acid fermentation was carried out with *A. niger* KCU520 using 14% sucrose solution of pH 2.0 as substrate. To each medium indicated amount of NH_4NO_3, K_2HPO_4 and $MgSO_4.7H_2O$ were added and fermentation was carried out at 30°C under high aerated conditions (2 l/min). After 15 days of fermentation citric acid was measured as described under Methods.

$MgSO_4.7H_2O$ (%)	NH_4NO_3 (%)																							
	0.01				0.1				0.25				0.5				1.0				3.0			
	K_2HPO_4 (%)																							
	0.0	0.01	0.1	1.0	0.0	0.01	0.1	1.0	0.0	0.01	0.1	1.0	0.0	0.01	0.1	1.0	0.0	0.01	0.1	1.0	0.0	0.01	0.1	1.0
	Carbohydrate conversion to citric acid (%)																							
0.01	5.0	48.2	37.8	23.5	6.2	61.3	48.2	36.4	6.7	60.5	80.6	53.1	5.8	56.1	78.2	48.1	7.2	42.1	60.1	45.1	5.8	32.5	48.7	50.1
0.03	6.7	51.2	42.6	26.4	6.1	63.2	50.1	39.1	6.1	63.1	84.3	62.1	6.8	60.2	81.9	50.9	7.5	45.6	62.5	51.3	7.2	35.4	51.3	56.2
0.05	6.1	51.6	34.2	22.1	6.8	58.4	49.3	40.2	6.3	62.7	83.1	63.2	7.1	58.7	75.3	50.3	6.8	43.2	61.9	50.7	6.3	36.7	50.9	52.4
0.1	6.5	46.1	35.7	20.7	5.8	59.1	46.7	36.1	5.9	60.5	79.9	60 .1	6.9	55.2	76.2	47.2	5.0	44.1	58.3	45.0	6.5	32.5	47.1	48.7

4.3 EFFECT OF SOME REGULATORS ON CITRIC ACID PRODUCTION BY *A. niger* MUTANT, KCU520 IN SUBMERGED AND SURFACE CULTURE

A number of regulators such as vegetable oils, H_2O_2, starch and air are known to have a marked influence on the citric acid formation by *A. niger* (Perlman and Sih, 1960; Kapoor et al., 1982; Rohr et al., 1983). Fig.21 shows the effect of different vegetable oils on citric acid biosynthesis and total cell mass in the submerged culture. While the citric acid formation was only marginally (5-10%) increased in presence of 1% (v/v) vegetable oils, there was a significant variance in the cell mass production. For instance, in the presence of butter fat, mustard oil or kardi oil the increase in the total cell mass was 33%, 60% and 33% more than in the controls without vegetable oils. In contrast, the total cell mass in the presence of groundnut oil and soybean oil was only 7% and 10% higher than the control. On the basis of citric acid to cell mass ratio, groundnut oil appeared to us, to be a better regulator than other oils. In order to determine the optimum concentration of groundnut oil, the effect of varying concentration of oil on the citric acid production by *A. niger* KCU520 was studied. As shown in Fig.22, the maximum effect was observed at 2% level of groundnut oil in the fermentation medium. At this concentration, the conversion of sugar into citric acid was about 87% and the increase in the total cell mass was 41% more.

Effect of H_2O_2 and starch on citric acid biosynthesis by *A. niger* KCU520 is shown in Fig.23 and 24, respectively. In both cases only a marginal increase (5-10%) in citric acid production was observed when fermentation was carried out in submerged culture with proper aeration. From these results it is clear that in submerged culture if the aeration is properly maintained then the effect of vegetable oils, H_2O_2 or starch on the production of citric acid by *A. niger* KCU520 is not well pronounced and as such use of these substrates would not be of much significance in the process development. In this respect, mutant *A. niger* KCU520 seems to us very promising for citric acid fermentation, since it works equally efficiently in absence of these regulators.

The effect of groundnut oil, H_2O_2 and starch was also studied in surface culture where formation of a floating cake of cells is an essential component. As was observed in the case of submerged culture, the effect of groundnut oil, H_2O_2 or starch, at optimum level, on the citric acid production during 15 days surface fermentation was about 8-10% more

FIG.21 EFFECT OF DIFFERENT VEGETABLE OILS ON CITRIC ACID FERMENTATION.

Submerged citric acid fermentation was carried out with *A. niger* KCU520 in the presence of various commercially available oils/fats. These oils/fats were added to the fermentation medium before sterilization, in the strength of 1%. Fermentation was carried out under optimum conditions for 15 days at 28°C in an orbital shaker at 150 rpm to ensure a moderate air supply. After fermentation, citric acid (□) and dry biomass (▨) were measured as described under Methods. C, B, F, S, G, M and K represents control, butter fat, soybean oil, groundnut oil, mustard oil and kardi oil, respectively.

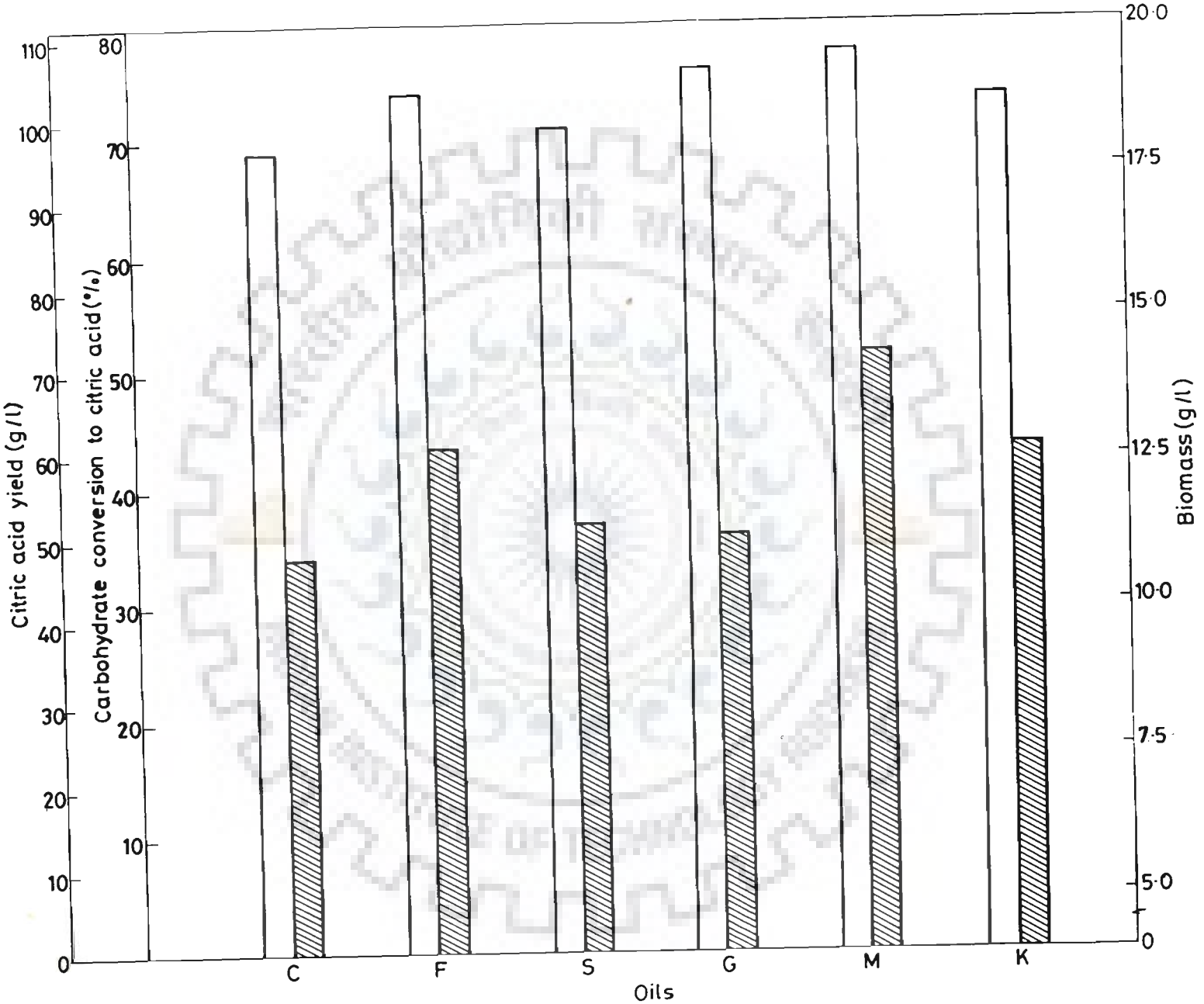

Citric acid yield (g/l)
Carbohydrate conversion to citric acid(%)
Biomass (g/l)
Oils
C
F
S
G
M
K
0
10
20
30
40
50
60
70
80
90
100
110
10
20
30
40
50
60
70
80
5·0
7·5
10·0
12·5
15·0
17·5
20·0

Fig. 21

FIG.22. EFFECT OF GROUNDNUT OIL ON CITRIC ACID FERMENTATION.

Submerged citric acid fermentation was carried out using *A. niger* KCU520 in presence of varying concentration of groundnut oil. Groundnut oil was added to the medium before sterilization. Fermentation was carried out under optimum conditions for 15 days at 28°C in an orbital shaker at 150 rpm to ensure a submerged culture under well aerated conditions. After completion of fermentation, citric acid (o——o) and dry biomass (Δ——Δ) were determined as described under Methods.

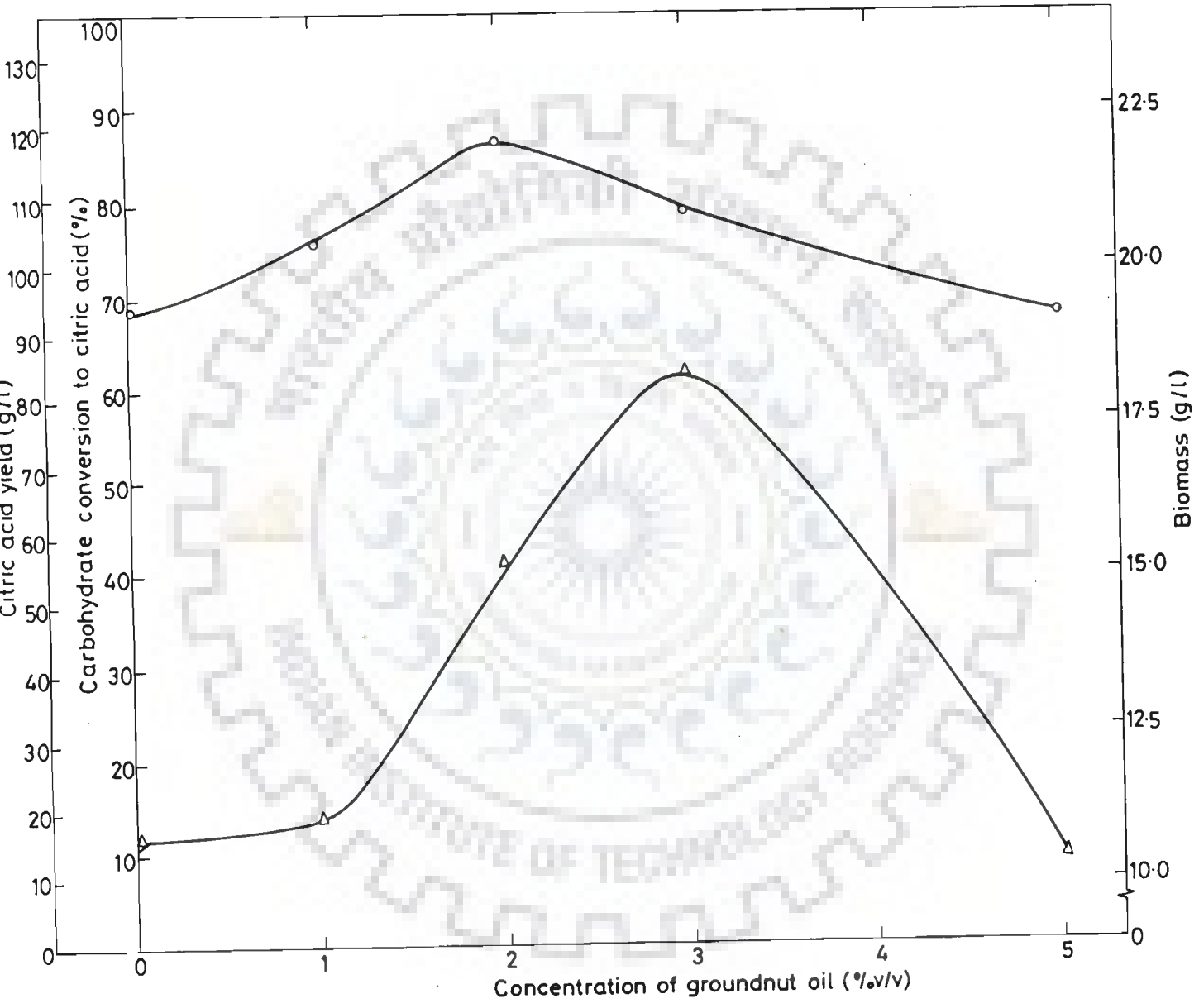

Citric acid yield (g/l)
Carbohydrate conversion to citric acid (%)
Biomass (g/l)
Concentration of groundnut oil (%v/v)
0
1
2
3
4
5
10
20
30
40
50
60
70
80
90
100
110
120
130
10·0
12·5
15·0
17·5
20·0
22·5

Fig. 22

FIG.23 EFFECT OF HYDROGEN PEROXIDE ON CITRIC ACID FERMENTATION.

Submerged citric acid fermentation was carried out using *A. niger* KCU520 in presence of varying concentration of H_2O_2. Addition of H_2O_2 to the medium was done prior to sterilization. Fermentation was done under optimum fermentation conditions in an orbital shaker at 150 rpm. After 15 days of fermentation, citric acid (o——o) and dry biomass (Δ——Δ) were detected as described under Methods.

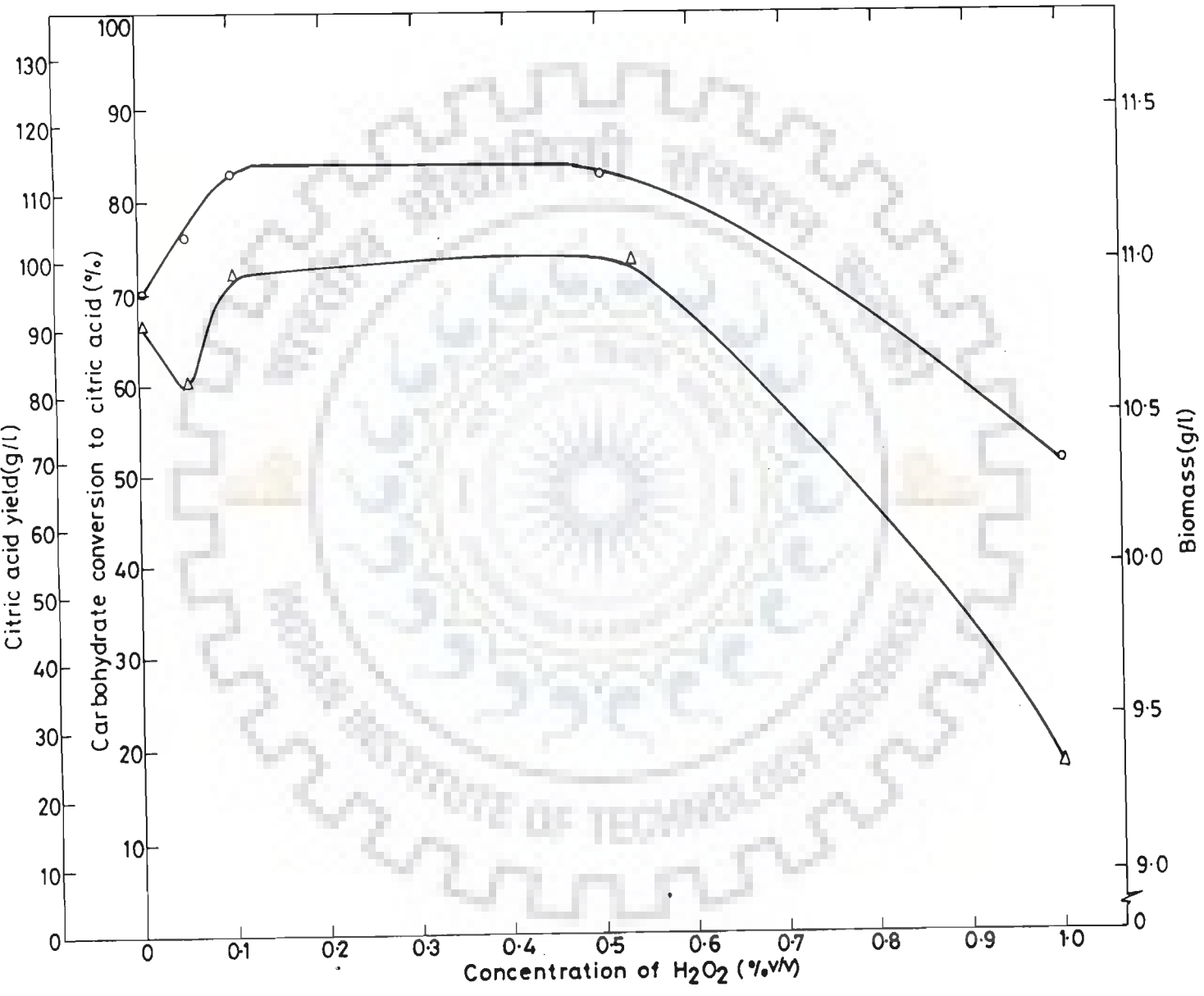

Citric acid yield(g/l)
Carbohydrate conversion to citric acid (%)
Biomass(g/l)
Concentration of H_2O_2 (%v/v)
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
9·0
9·5
10·0
10·5
11·0
11·5

Fig. 23

FIG.24 EFFECT OF STARCH ON CITRIC ACID FERMENTATION.

Submerged citric acid fermentation was carried out using *A. niger* KCU520 in presence of varying concentration of starch. Desired amount of starch was added to the fermentation medium before sterilization. Fermentation was done at 28°C in an orbital shaker at 150 rpm . Citric acid (o——o) and dry biomass (Δ——Δ) were measured after continuous fermentation of 15 days, as described under Methods.

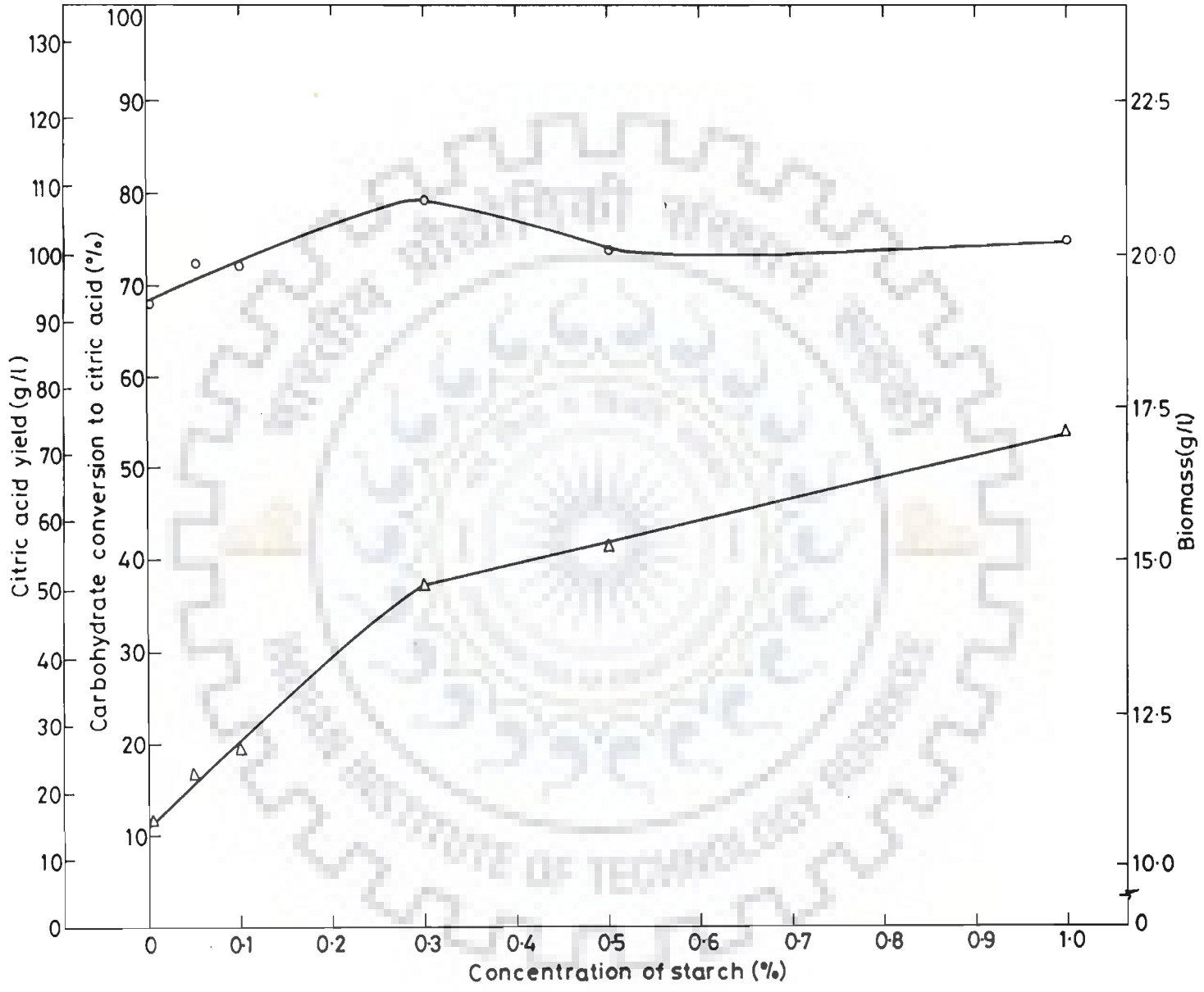

Citric acid yield (g/l)
Carbohydrate conversion to citric acid (%)
Biomass(g/l)
Concentration of starch (%)
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
10·0
12·5
15·0
17·5
20·0
22·5

Fig. 24

than that of the control where no regulator was used (Fig.25). In addition, by aerating the culture flasks well, nearly 2 fold increase in citric acid yield was observed showing that air is essential for citric acid fermentation. Furthermore, the pattern of citric acid production during fermentation in the presence or absence of groundnut oil, H_2O_2 and starch was identical.

A comparison of citric acid formation by *A. niger* KCU520 in surface and submerged cultures with and without groundnut oil, H_2O_2 or starch under an excess air (forced air circulation at the rate of 2 l/min through the medium or over the surface) supply is given in Table-2. To see the effect of air, citric acid fermentation was also performed in absence of air. It can be seen that the yield of citric acid in each case was 8-10% higher in surface culture than that of submerged culture. In addition, similar increase in citric acid yield was observed when either groundnut oil (2%) or H_2O_2 (0.1%) or starch (0.3%) was added in the fermentation medium. However, it was observed that in absence of air the citric acid production was reduced to half.

4.4 USE OF SUGARCANE MOLASSES FOR CITRIC ACID PRODUCTION BY FERMENTATION BY *A. niger* KCU520

After optimizing the citric acid fermentation using sucrose as carbon source, sugarcane molasses was tried for citric acid fermentation using *A. niger* KCU520. Some details about the composition of sugarcane molasses used in this study are given below:

4.4.1 Composition of sugarcane molasses

The composition of sugarcane molasses used in this study is shown in Table-3 . Carbohydrate component accounts for about 50% of which 70 to 75% is sucrose. Thus the latter component accounts for nearly one- third of the total weight of molasses which cannot be separated by crystallization. Besides carbohydrate, sugarcane molasses was found to contain about 2% protein and 12-13% sulphated ash. Potassium accounts for 2.5 to 4.0% and phosphorus content varies between 1 and 2%. Among the trace metals, Fe

FIG.25 EFFECT OF VARIOUS REGULATORS ON CITRIC ACID FERMENTATION IN SURFACE CULTURE.

Surface citric acid fermentation was carried out with *A. niger* KCU520 in presence of 2% groundnut oil, 0.1% H_2O_2 or 0.3% starch under optimum fermentation conditions. For the sake of comparison, surface fermentation was also carried out in absence of the above mentioned regulators and also without any extra supply of air to the fermentation system. All regulators were added to the medium before sterilization. Fermentation was terminated by autoclaving at a desired time interval and citric acid and dry biomass were determined as described under Methods. (o—o), (Δ—Δ), (●—●), (□—□) and (X—X) shows the citric acid production in presence of high aeration, high aeration + 2.0% groundnut oil, high aeration + 0.3 % starch, high aeration + 0.1% H_2O_2 and without extra air supply, respectively.

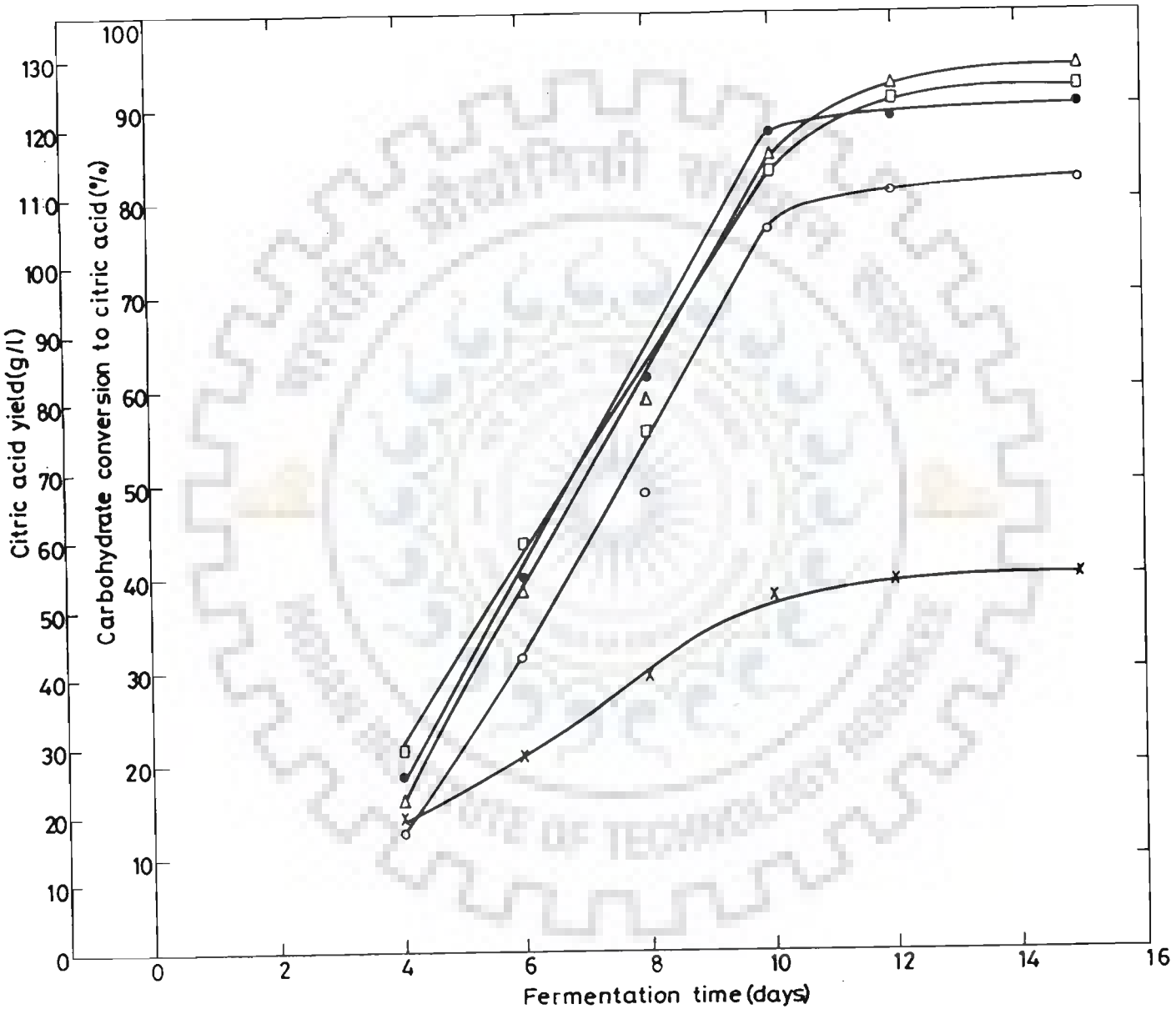

Citric acid yield(g/l)
Carbohydrate conversion to citric acid (%)
Fermentation time (days)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
0
2
4
6
8
10
12
14
16

Fig. 25

TABLE-2

EFFECT OF VARIOUS REGULATORS ON CITRIC ACID FERMENTATION BY *A. niger* KCU520

Regulator	Optimum concentration %, v/v	Yield of Citric Acid (%)	
		Surface Culture[1]	Submerged Culture[2]
Control		82.3	69.0
Groundnut Oil	2.O	94.2	86.8
Hydrogen-peroxide	0.1	93.8	84.6
Starch	0.3	89.7	79.1
Control[3](without extra air supply)	-	39.0	31.2

1. Sterilized air was circulated over the surface of the cell mass cake at a rate of 2 lit/min with the help of an air compressor or by sucking the air with the help of a suction pump.

2. Aeration was provided by shaking the culture flask in an orbital shaker at 150 rpm.

3. Extra air supply was stopped by eliminating any aeration facility in surface culture and plugging the culture flasks with stoppers in case of submerged fermentation.

TABLE -3 COMPOSITION OF SUGARCANE MOLASSES[1]

Component	%[3]
Total carbohydrates	50.0-53.0
Sucrose	35.0-37.0
Protein	1.8-2.4
Non-sugar organic matter	10.0-11.5
Sulphated ash	12.0-13.0
Sodium	0.1-0.3
Potassium	2.5-4.0
Calcium	0.5-0.8
Phosphorus	1.0-2.0
Copper	0.0005-0.0015
Iron	0.03-0.04
Zinc	0.00075-0.002
Manganese	0.0005-0.001
Vitamins and others	0.0006-0.00075
Water[2]	22.5-28.0

1. Sugarcane molasses was obtained from Iqbalpur (Distt. Saharanpur, India) sugar factory during the months February and March when the sugarcane was fully mature.

2. Water values were by difference.

3. Values are average of atleast triplicate samples from at least 3- 4 batches of sugarcane molasses.

content vary from 0.03-0.04%, Cu and Mn were 0.0005 to 0.001% each and Zn was present upto 0.002%.

4.4.2 Clarification of sugarcane molasses by hexacyanoferrate (HCF) for citric acid production

Crude sugarcane molasses has been reported to contain some inhibitors, especially heavy metal (Cu, Fe, Zn and Mn) ions which interfere in the citric acid fermentation process. Hence, removal of these metal ions is an essential requirement before molasses can be used as a substrate for production of citric acid by fermentation, using *A. niger*. Since it is already known that HCF-treatment precipitates almost all metals from molasses (Clark et al., 1965), HCF-treatment was tried in the present study. Preliminary experiments showed that for the complete precipitation reaction, 15 min heating was sufficient at 70-90^{o}C, and that removal of metal ions can be efficiently done in a pH range between 3.0 and 5.0. Unless stated otherwise, HCF-treatment of molasses was done at pH 4.5. Under these conditions when diluted molasses, containing 12% sugars was treated with 0.1% HCF (w/v), about 88% Cu, 96% Fe, 95% Zn and 98% Mn ions were precipitated (Table-4). Since no more metals were precipitated at concentrations higher than 0.1% HCF, treatment of molasses with higher concentration of HCF was found to be unnecessary. This is also indicated by the fact that the molasses obtained after 0.1% HCF-treatment at pH 4.5 (treatment F) gave a much higher yield of citric acid by surface fermentation with a drastic reduction in cell mass formation (Fig.26). It is apparent that the amount of cell mass produced during the fermentation of HCF- treated molasses declined with increasing concentration of HCF used for treatment. This effect becomes more pronounced when HCF at or over 0.1% concentration is used. On the other hand, there is a gradual increase in citric acid concentration, i.e., conversion of sugars into citric acid, in treatment upto 0.1% HCF. Thereafter, the yield of citric acid starts to decline when higher concentration of HCF were employed. In fact 0.150% HCF-treated molasses yielded just 8% citric acid. In contrast, 0.1% HCF-treated molasses yielded 70% citric acid, which is comparable with the yield obtained when pure sucrose was used as substrate. It seems likely that higher concentration of HCF strongly inhibits cell growth, due to which the critical cell mass required for citric acid formation is not formed. Consequently the formation of citric acid is also limited. From these results the following conclusions are drawn :

TABLE - 4

The sugarcane molasses was diluted with deionized water to adjust the final concentration of sugars to 12%. Routinely 4.5x dilution of the crude molasses was done. After adjusting the pH 4.0-4.5, the metal ions were precipitated by the addition of indicated amounts of hexacyanoferrate (HCF) followed by heating at 70-90°C for 15 min. The precipitate was removed by filtration and the filtrate was analyzed for metal ions by atomic absorption spectrophotometer as described under methods. Results are expressed as per cent metal ions precipitated using metal concentration in dilute, untreated molasses as 100% un-precipitated. Values are average of atleast 3 determinations.

PRECIPITATION OF Cu^{2+}, Fe^{3+}, Zn^{2+} and Mn^{2+} FROM SUGARCANE MOLASSES BY HEXACYANOFERRATE TREATMENT

HCF Conc.	Metal ions precipitated by HCF							
(%)	Cu^{2+}	ppm	Fe^{3+}	ppm	Zn^{2+}	ppm	Mn^{2+}	ppm
0.000	0	0.00	0	0.00	0	0.0	0	0.0
0.025	59	1.18	53	37.10	52	1.3	60	0.9
0.050	74	1.48	66	46.20	67	1.67	70	1.14
0.075	81	1.62	82	57.40	82	2.05	90	1.35
0.100	88	1.76	96	67.20	95	2.37	98	1.47
0.125	90	1.80	97	67.90	96	2.40	98	1.47
0.150	90	1.80	96	67.20	95	2.37	89	1.45
0.175	87	1.74	95	66.50	96	2.40	98	1.47
0.200	88	1.76	94	65.80	95	2.37	96	1.44

FIG.26. OPTIMUM HCF CONCENTRATION FOR PRETREATMENT OF SUGARCANE MOLASSES FOR CITRIC ACID FERMENTATION BY A. niger MUTANT KCU520.

The crude sugarcane molasses was diluted with deionized water to adjust the sugar concentration to 12% and then subjected to clarification using different concentration of HCF as described under Methods. The HCF-treated molasses was then subjected to citric acid fermentation. The fermentation was carried out for 15 days in a surface culture under optimum conditions of pH, temperature and aeration. At the end of fermentation, amounts of citric acid (☐) and the dry biomass (▨) were determined by standard procedures. A, B, C, D, E, F, G and H represent control with sucrose, untreated molasses and 0.025%, 0.05%, 0.075%, 0.10%, 0.125% and 0.15% HCF-treated molasses, respectively.

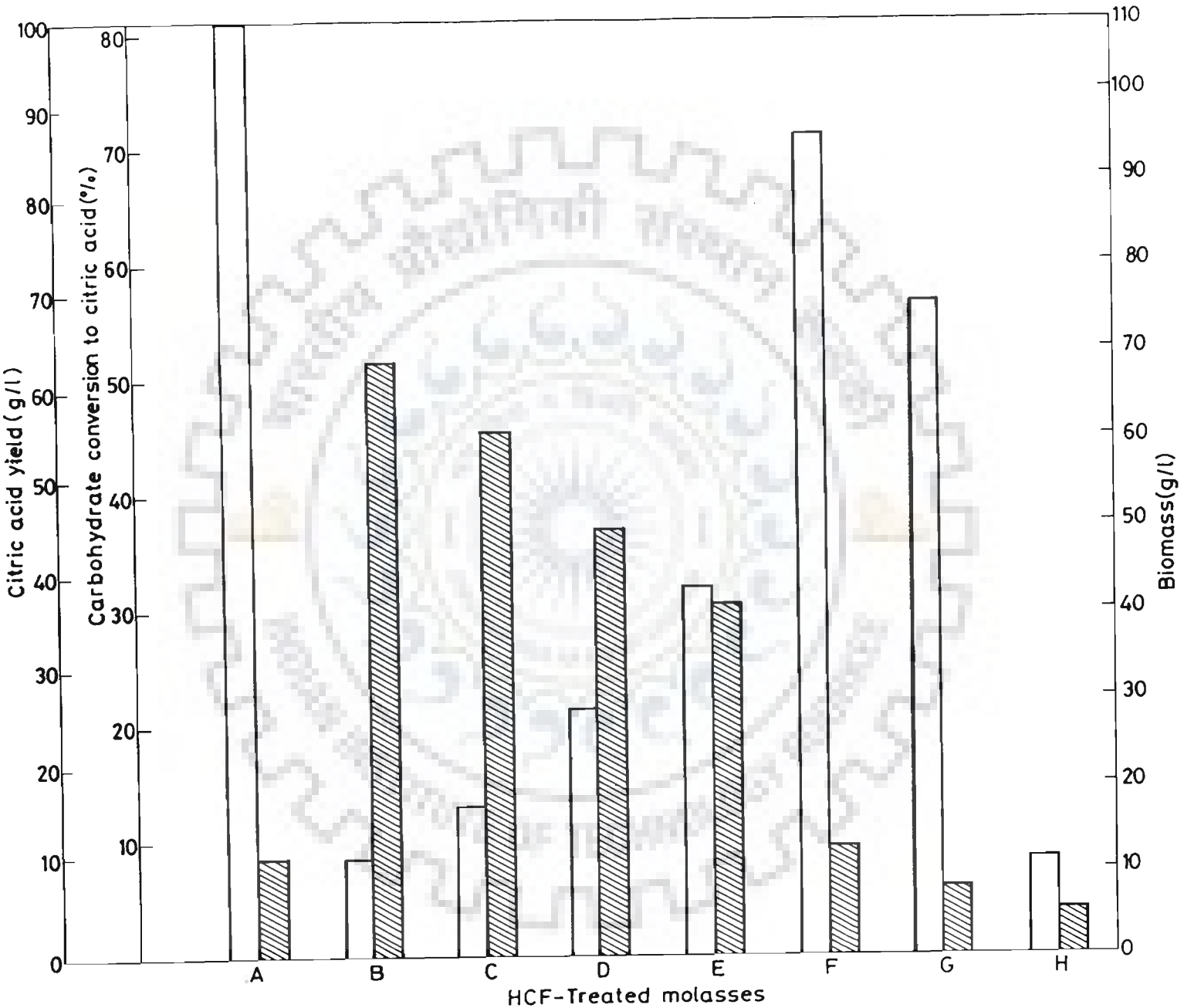

Citric acid yield (g/l)
Carbohydrate conversion to citric acid (%)
Biomass (g/l)
HCF-Treated molasses
A
B
C
D
E
F
G
H

Fig. 26

(i) Metal ions (Cu^{2+}, Fe^{3+}, Zn^{2+}, and Mn^{2+}), when present above a critical concentration (in the present case Cu^{2+} = 0.24 ppm; Fe^{3+} = 2.8 ppm; Zn^{2+} = 0.13 ppm and Mn^{2+} = 0.03 ppm) seem to support the cell growth at the cost of citric acid production and

(ii) 0.1% HCF-treated molasses is ideal for citric acid production. However, the latter conclusion is subject to quality and type of molasses used.

4.4.3 Effect of pH on citric acid formation by fermentation of HCF- treated sugarcane molasses

Fig.27 shows the effect of pH on the production of citric acid by fermentation of 0.1% HCF-treated molasses at pH 4.5. The pH of molasses was adjusted with 1 N HCl and different concentrations of sugar in molasses were adjusted by proper dilution with deionized water. Fermentation was carried out for 10 days at 28°C using *A. niger* KCU520 strain in surface culture. Results shown in Fig.27 show that pH optima is dependent on the sugar content of molasses. For example, in molasses containing 8-10% sugars, the optimum pH for citric acid fermentation was 4.0, whereas when the sugar concentration of molasses was 12% and 15% the pH optima was found to be 4.5 and 5.0, respectively. In addition, it was found that maximum citric acid was formed by fermentation in surface culture with *A. niger* KCU520 strain when the sugar concentration of molasses was around 12% and treated with 0.1% HCF at pH 4.5 and 70-90°C for 15 min. The molasses after HCF-treatment was referred to as clarified molasses.

4.4.4 Effect of phosphate on HCF-treated molasses for citric acid fermentation

Fig.28 shows the effect of phosphate on citric acid production and the relationship between cell mass and citric acid production. It was found that in the absence of exogenous supply of inorganic phosphate to the fermentation medium, yields of citric acid and the cell mass were only 23% and 3.5 mg/ml, respectively. But when the medium was supplemented with phosphate (0.1% K_2HPO_4), the citric acid yield increased three folds and so was the case

FIG.27 EFFECT OF pH ON CITRIC ACID PRODUCTION BY A. niger KCU520 IN SURFACE FERMENTATION.

The HCF clarified molasses was used as a substrate for citric acid production by surface fermentation at different pH's. The pH of the medium was adjusted with 1N HCl and the fermentation was carried out for 15 days under optimum conditions. The citric acid was determined as described under Methods.(o—o), (Δ—Δ), (□—□) and (●—●) represent the amount of citric acid produced by fermentation of HCF-treated molasses containing 8, 10, 12 and 15% sugars, respectively.

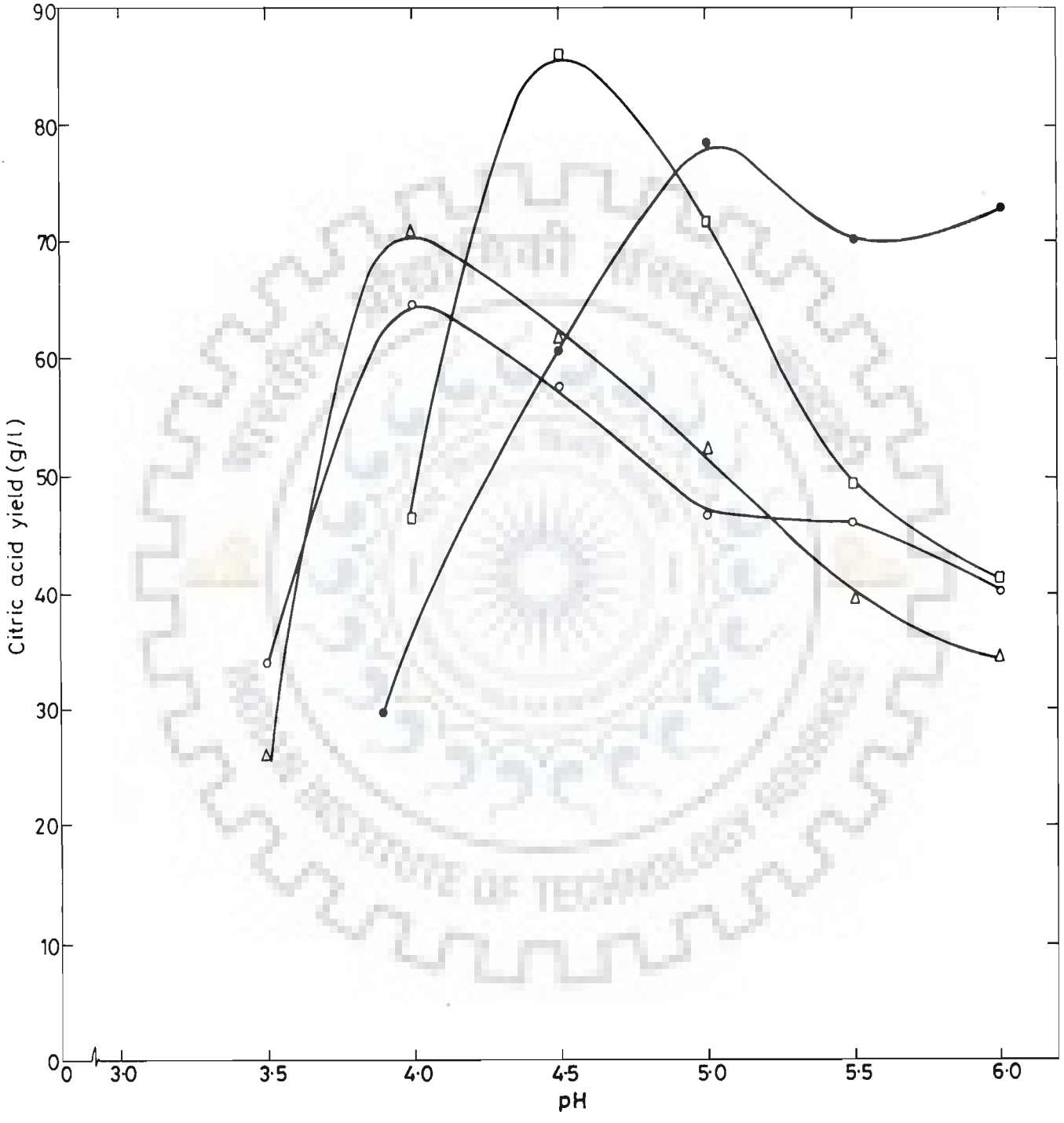

90
80
70
60
50
40
30
20
10
0
Citric acid yield (g/l)
0
3·0
3·5
4·0
4·5
5·0
5·5
6·0
pH

Fig. 27

FIG.28 EFFECT OF INORGANIC PHOSPHATE (K_2HPO_4) ON CITRIC ACID FERMENTATION BY A. niger KCU520.

Surface fermentation was carried out for 15 days under optimum conditions using varying concentrations of K_2HPO_4 in the fermentation medium. 0.1% or 0.15% HCF-treated sugarcane molasses was used as substrate. Amount of citric acid (g/l) (o——o) and dry biomass (Δ——Δ) were determined as described under Methods.

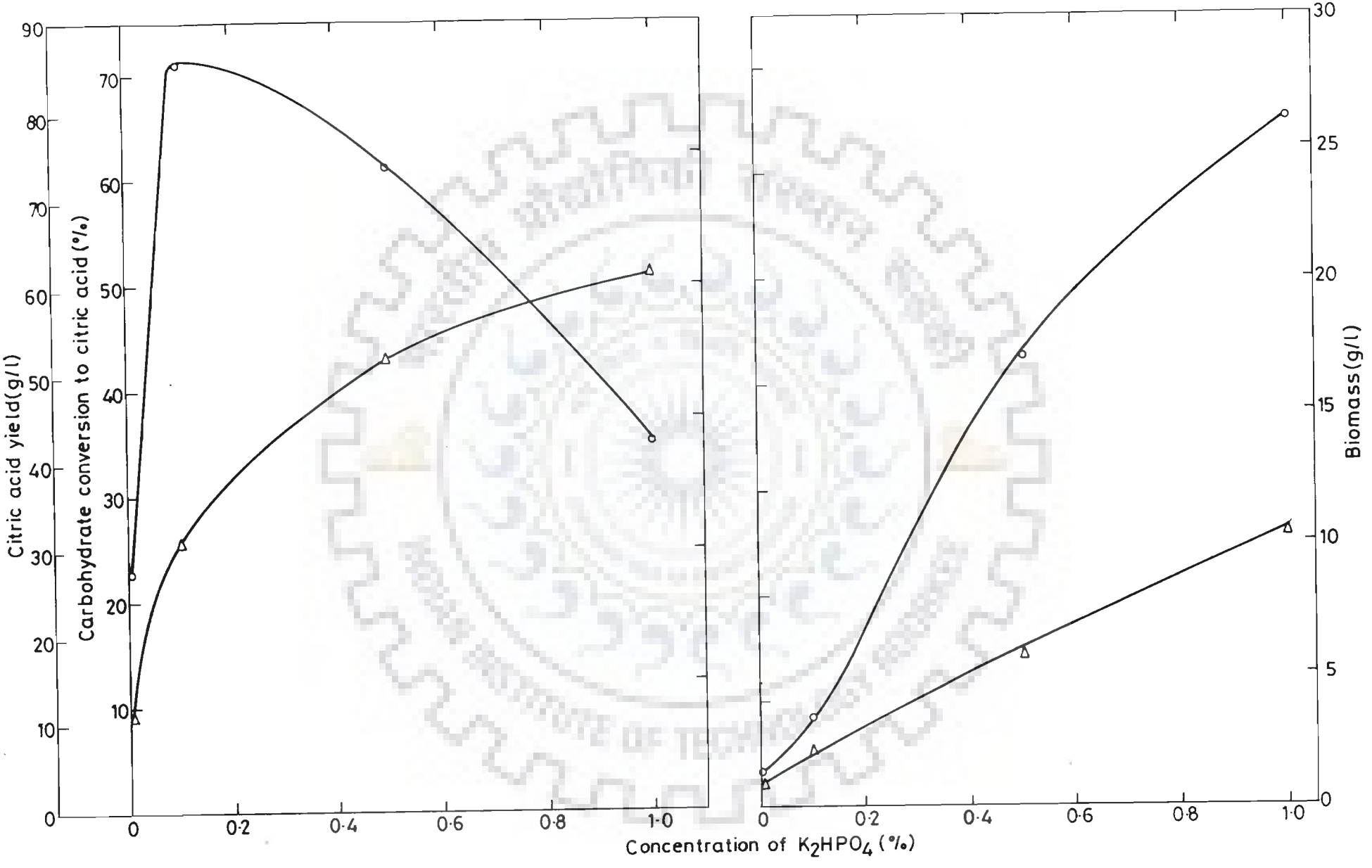

Citric acid yield(g/l)
Carbohydrate conversion to citric acid (%)
Biomass (g/l)
Concentration of K_2HPO_4 (%)
0
0·2
0·4
0·6
0·8
1·0
10
20
30
40
50
60
70
80
90
5
15
25

Fig. 28

with biomass. In contrast, however, beyond 0.1% K_2HPO_4 the citric acid yield started to decline but the total biomass was still increasing, presumably at the cost of citric acid production by utilizing the substrate. From these results, it is obvious that a minimum level of inorganic phosphate is essential for optimum production of citric acid by fermentation. The fermentation medium containing clarified molasses, was therefore, routinely supplemented with K_2HPO_4 to a final concentration of 0.1% to sustain proper cell growth required for optimum production of citric acid. Addition of phosphate to the fermentation medium was also found to relieve the inhibitory effect of HCF on cell growth and subsequently on citric acid formation as can be seen from the results shown in Fig.28. The cell growth and citric acid formation, which were negligible when molasses clarified with 0.15% HCF was used as substrate, were greatly enhanced by phosphate supplementation (Fig.28). Infact, in the present case, it was possible to increase the citric acid yield from merely 3% to about 65% by the addition of K_2HPO_4 to a 1% level in the fermentation medium. These results indicate that high concentration of phosphate is able to counteract the inhibition of the fungal growth brought about by HCF.

4.4.5 Production of citric acid using various fermentation systems

Submerged, surface and solid state surface fermentation systems were tried to prepare citric acid from the clarified sugarcane molasses. Fig.29 shows the time course for citric acid production during various fermentation systems under optimum conditions. In all cases, 10-12 days period was found optimum for the completion of fermentation. After this period, nearly 65%, 75% and 80-85% of carbohydrates (molasses sugar) were converted to citric acid in submerged, surface and solid state fermentations, respectively. These results show that both surface and solid state fermentations are quite promising.

4.4.6 Further improvement in citric acid yield during solid state surface fermentation

In an attempt to improve the yield of citric acid in SSF, effect of various regulators, namely groundnut oil, starch and H_2O_2 was studied. A marginal increase (about 5%) in citric acid production was observed when SSF was performed in presence of 0.1% H_2O_2, 2%

FIG.29 COMPARISON OF CITRIC ACID PRODUCTION IN SUBMERGED, SURFACE AND SOLID STATE SURFACE FERMENTATIONS BY A. niger KCU520.

Submerged, surface and solid state surface systems were used for citric acid production by *A. niger* KCU520 under optimum conditions. The amount of citric acid produced in different time period is expressed in g/l. (□——□), (○——○) and (Δ——Δ) shows citric acid produced in submerged, surface and solid state surface fermentation systems, respectively.

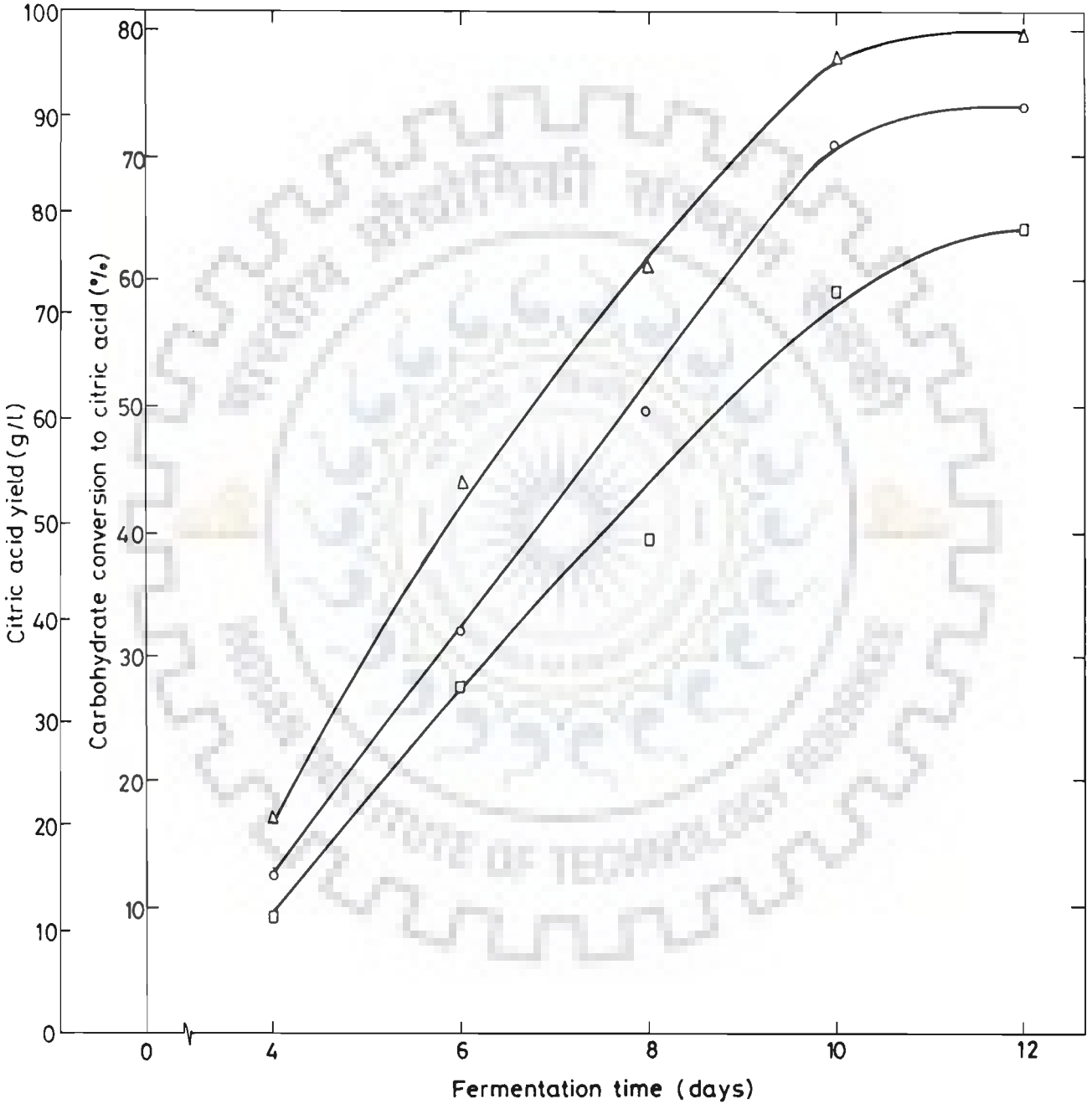

Citric acid yield (g/l)
Carbohydrate conversion to citric acid (%)
Fermentation time (days)
0
4
6
8
10
12
10
20
30
40
50
60
70
80
90
100

Fig. 29

groundnut oil or 0.3% starch in the fermentation medium (Fig.30). The slight improvement in citric acid production in presence of groundnut oil or starch was insignificant and does not seem to be of any practical importance. It is clear from Fig.30 that continuous surface circulation of air is absolutely essential for a better yield of citric acid. In this regard, H_2O_2 could possibly have an added advantage of providing the much needed aeration for citric acid fermentation. The overall effect of H_2O_2 in SSF seems to be comparable to that of simple surface culture.

4.4.7 Scaling-up of fermentation process of citric acid production from molasses by solid state surface fermentation

The citric acid fermentation was scaled-up to use 10 liters of molasses. A simple fermenter was designed (Fig.3), based on the findings for solid state fermentation or surface fermentation. Circular stainless steel trays (30 x 30 x 3 cm) were used to hold fermentation medium, etc. To each tray was added 0.75, 1.0, 1.25 and 1.5 liters fermentation medium and 15% powdered bagasse which corresponded to 1.4, 1.8, 2.2 and 2.6 cms of medium depth in trays. The results of per cent carbohydrate converted to citric acid under optimum conditions for fermentation in SSF are given in Fig.31. It was found that depth of the fermentation medium (when the total surface area of the tray is fixed) had a significant influence on over all production of citric acid, presumably because of the contact between the medium and the culture. Best results were obtained when the ratio of surface area of the base of tray to the medium depth was about 400. Hence, in all experiments the diameter of trays was fixed and fermentation medium was filled such that the above mentioned base surface to medium depth ratio could be achieved.

From the previous study, H_2O_2 seems to be of importance and therefore it was tried in the large scale SSF process. The combined effect of H_2O_2 and excess of air supply is shown in Fig.32. As can be seen from these results, there is no significant difference in the citric acid production whether or not H_2O_2 is added to the medium. This might be due to the availability of large surface area for better aeration in large scale fermentation which subsides the effect of H_2O_2. However, the yield of citric acid in the presence of limited air supply was nearly half. Putting all the observations together it is concluded that in SSF the air circulation to the medium is essential and can not be totally replaced by H_2O_2.

FIG.30 EFFECT OF SOME REGULATORS ON CITRIC ACID FERMENTATION.

Solid state surface fermentation was carried out in the presence of 2% groundnut oil, 0.1% H_2O_2 or 0.3% starch for indicated periods, under optimum fermentation conditions. All regulators were added to the medium before sterilization. After an indicated period of time, the fermentation was stopped and citric acid was measured according to Methods. (o——o), (●——●), (Δ——Δ), (□——□) and (X——X) shows citric acid production in presence of high aeration, high aeration + 0.3% starch, high aeration + 2.0% groundnut oil, high aeration + 0.1% H_2O_2 and without extra air supply, respectively.

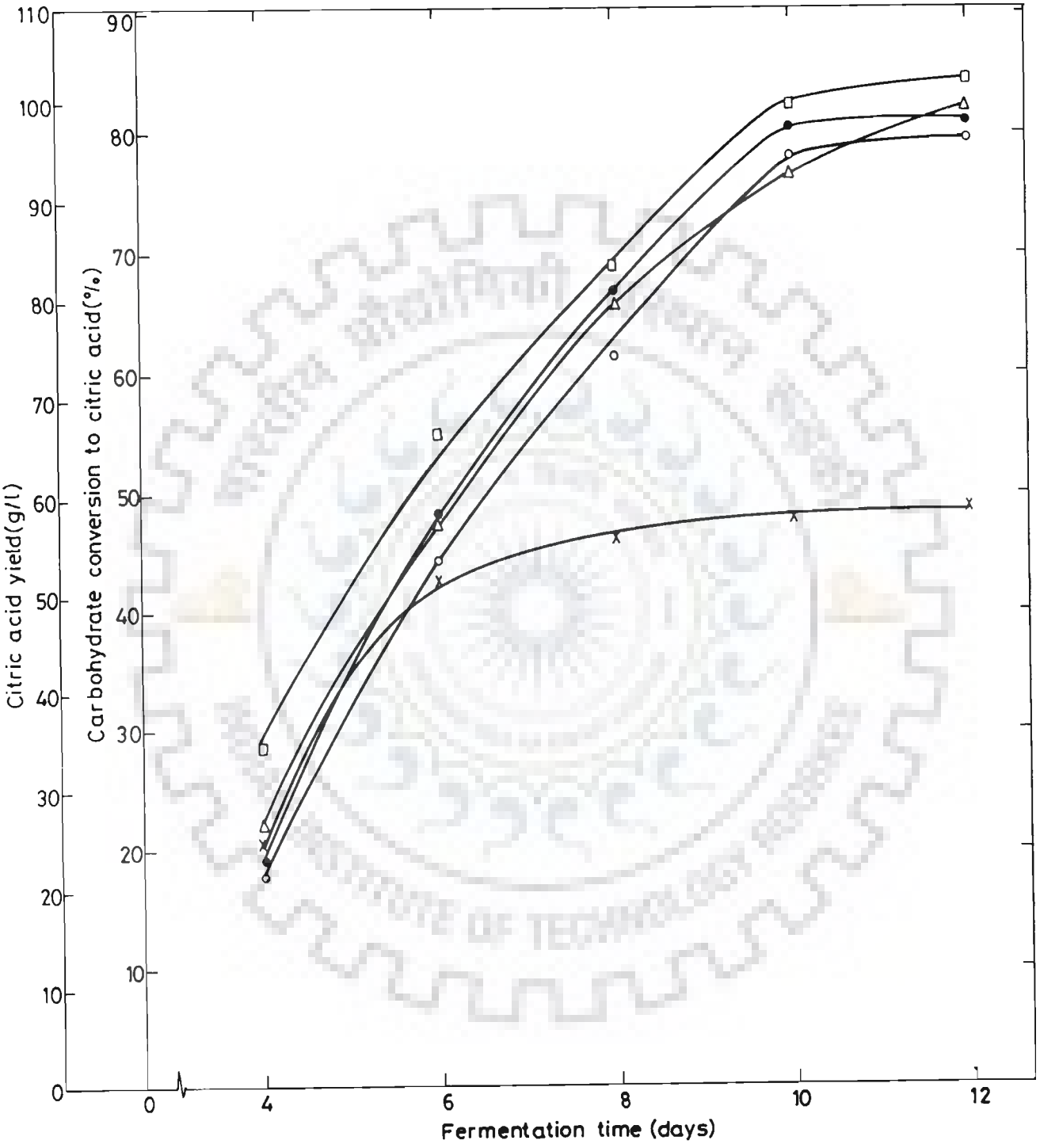

Citric acid yield(g/l)
Carbohydrate conversion to citric acid(%)
Fermentation time (days)
0
4
6
8
10
12
10
20
30
40
50
60
70
80
90
100
110

Fig. 30

FIG.31 EFFECT OF MEDIUM DEPTH ON CITRIC ACID PRODUCTION.

Large scale solid state surface fermentation was carried out with *A. niger* KCU520 in stainless steel plates using a 10 litres surface fermenter as described under Methods. 0.1% HCF-treated molasses containing 12% carbohydrates at pH 4.5 was used as substrate. To each tray was added 0.75, 1.0, 1.25 and 1.5 litres molasses medium mixed with 15% bagasse (70% moisture contents), to obtain a medium depth of 1.4, 1.8, 2.2 and 2.6 cm, respectively. Fermentation was carried out for 10 days at 28°C under high aerated conditions. The amount of citric acid formed was determined as described under Methods.

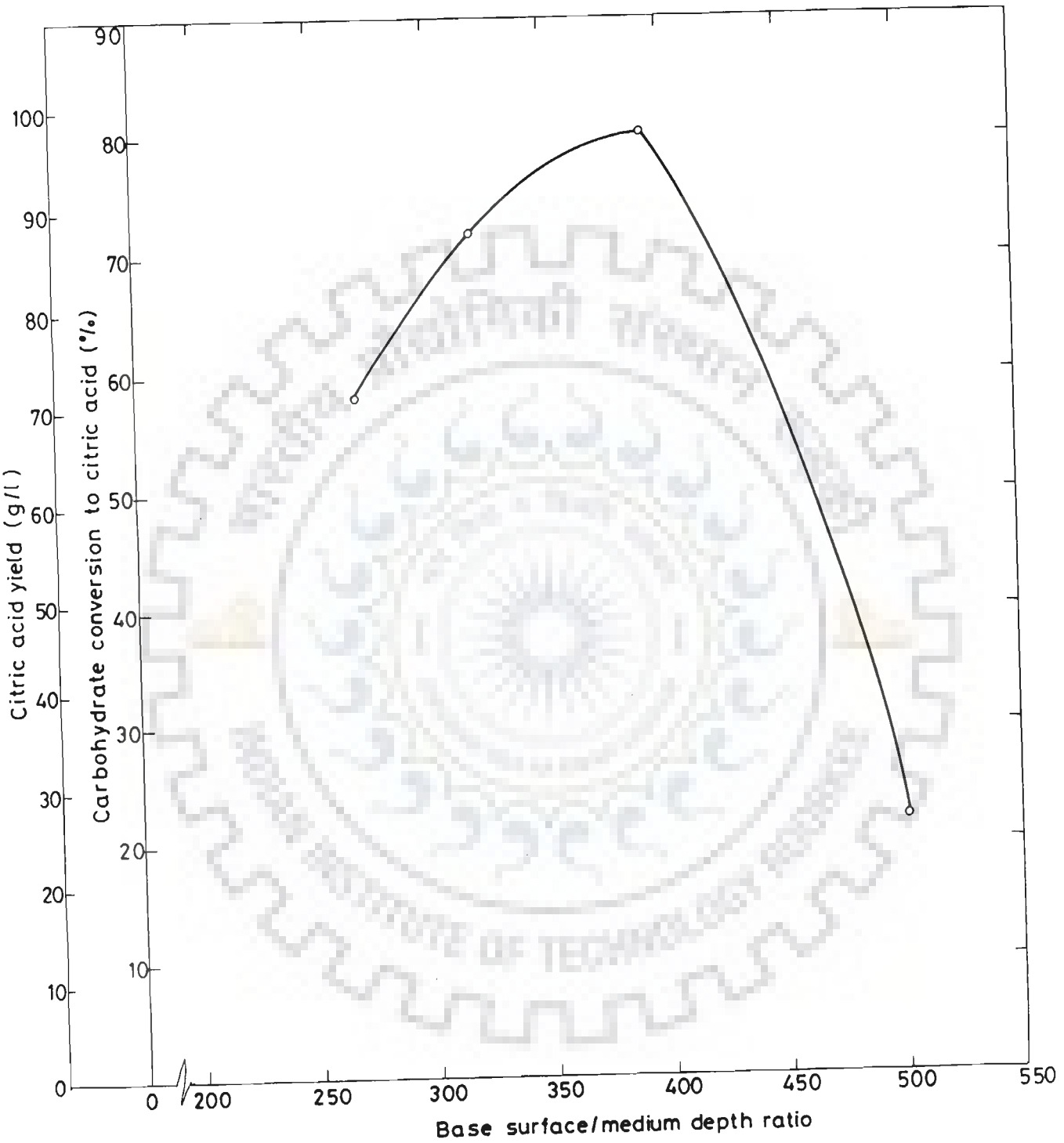

Citric acid yield (g/l)
Carbohydrate conversion to citric acid (%)
Base surface/medium depth ratio
0
10
20
30
40
50
60
70
80
90
100
200
250
300
350
400
450
500
550

Fig. 31

FIG.32 LARGE SCALE PRODUCTION OF CITRIC ACID USING SOLID STATE SURFACE FERMENTATION.

Solid state surface fermentation was carried out using *A. niger* KCU520 in a 10 litres surface fermenter under optimum fermentation conditions as described under Methods. Fermentation was carried out in the presence of 0.1% H_2O_2. Parallel experiment was also performed in which large scale SSF was done without supplying extra air to the cultures. After indicated period, fermentation was stopped followed by measurement of citric acid in fermented broth as described under Methods. (o—o), (□—□) and (X—X) shows the citric acid production in presence of high aeration, high aeration + 0.1% H_2O_2 and in absence of extra air supply, respectively.

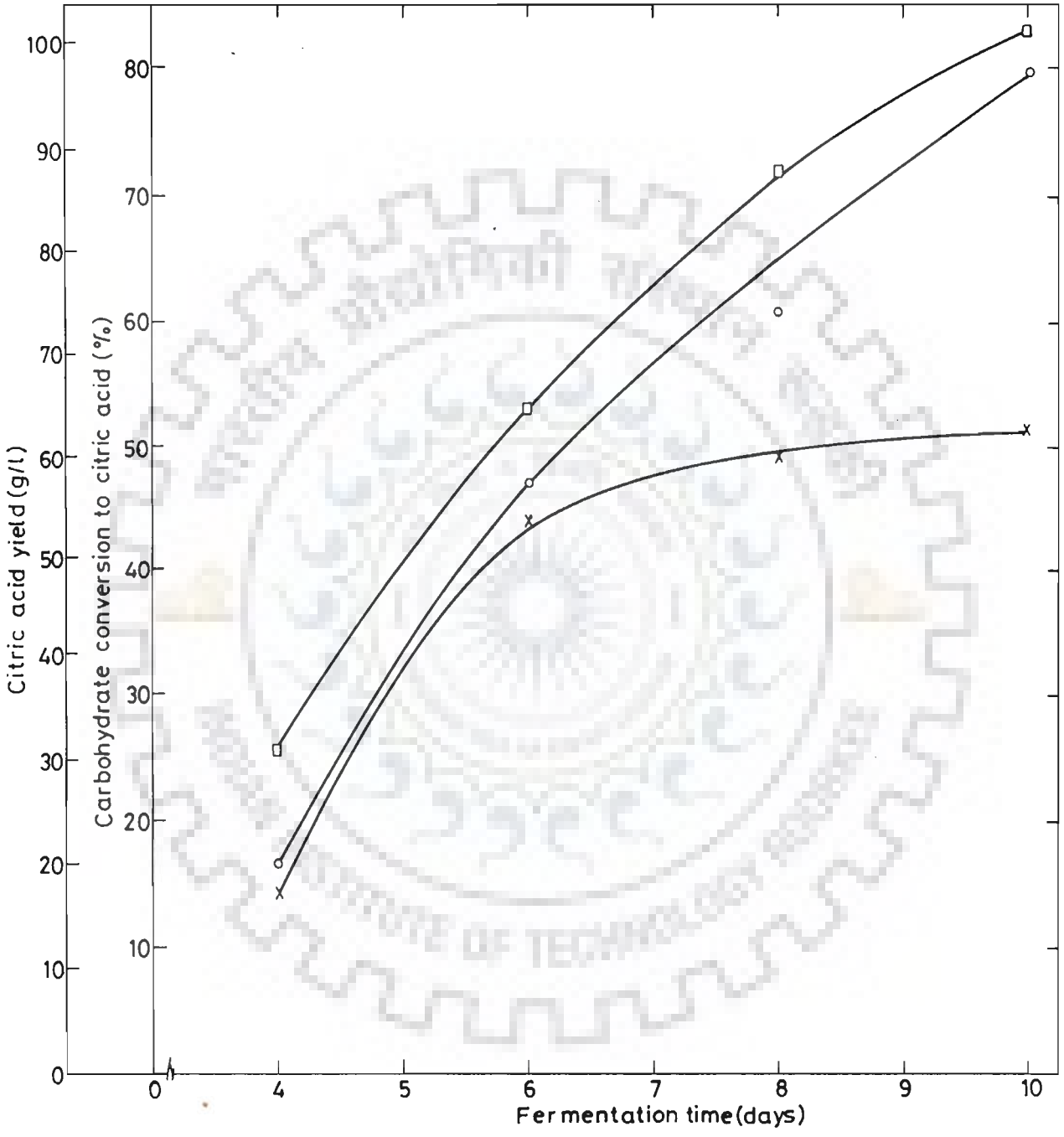

Citric acid yield (g/l)
Carbohydrate conversion to citric acid (%)
Fermentation time(days)
0
10
20
30
40
50
60
70
80
90
100
4
5
6
7
8
9
10

Fig. 32

4.4.8 Use of solid state surface fermentation for multiple fermentation cycle

Experiments were designed to use SSF for multiple fermentation cycles, i.e., using the same *A. niger* KCU520 culture bed, representing pseudoimmobilization of *A. niger* mycelium on cellulose fibers (bagasse), for repeated fermentation by replacing the fermentation medium, after appropriate accumulation of citric acid, by the fresh medium. Earlier experiments had shown that the first 5-days of fermentation are a kind of growth phase following which the mycelium mature to produce citric acid from sugar more efficiently. At this stage (after 5-days of fermentation), when nearly 40% of carbohydrates were converted to citric acid (Fig.33), the fermentation medium was drained-out without disturbing the mycelium bed and fresh medium was fed into the system for a second cycle of fermentation. Interestingly enough, about 80% of sugars were converted to citric acid within 5- days of fermentation, whereas under standard conditions with fresh inoculum, it took 10-days to achieve this yield (Fig.29). Hence, the fermentation period was cut down to nearly half. In addition, a third fermentation cycle was also performed with equal efficiency (Fig.33). After the 3rd cycle, the citric acid fermentation efficiency starts to decline. These results clearly indicate the potentiality of SSF process for large scale production of citric acid from molasses, the main advantage being as follows: **(i) the yield is better (about 80% conversion of carbohydrate to citric acid), (ii) multiple cycles (3 to 4) can be performed without changing the culture bed acting as pseudoimmobilized system, and (iii) most importantly the fermentation period is reduced from 10 days to 5 days which would cut-down the cost of production tremendously.** Further research on a scaled-up system would be needed to establish the technical operation.

4.5 USE OF IMMOBILIZED *A. niger* KCU520 FOR CITRIC ACID PRODUCTION

So far batch process was used for citric acid fermentation. The results of pseudoimmobilization during SSF were encouraging and suggested that immobilization would come in handy for the development of continuous process.

FIG.33 USE OF SOLID STATE SURFACE FERMENTATION SYSTEM FOR MULTIPLE FERMENTATION CYCLES FOR ENHANCED CITRIC ACID PRODUCTION.

The solid state surface fermentation was carried out in 10x250 ml conical flasks fitted with an outlet/inlet at the bottom (see fig.2). 50 ml of HCF-treated sugarcane molasses containing 12% sugars was mixed with 15 g powdered bagasse and inoculated with *A. niger* KCU520. Fermentation was allowed for 5 days (one cycle) under optimum conditions of temperature (28°C), pH (4.5) and aeration (2 l/min). The fermented broth was removed by draining and fresh sterilized fermentation medium was introduced into the flask through the outlet/inlet arm. The fermentation was continued for another 5 days cycle (IInd-cycle). This process was repeated 6x, i.e., 6 fermentation cycles (each cycle of 5 days duration) were completed. The amount of citric acid formed in each fermentation cycle was estimated as described under Methods. An average rate (g/day) of citric acid production was determined by dividing the total amount of citric acid by 5. (☐) total citric acid per cycle and ; (▨) average rate (g/day) of citric acid formation. Values are average of 10 fermentations.

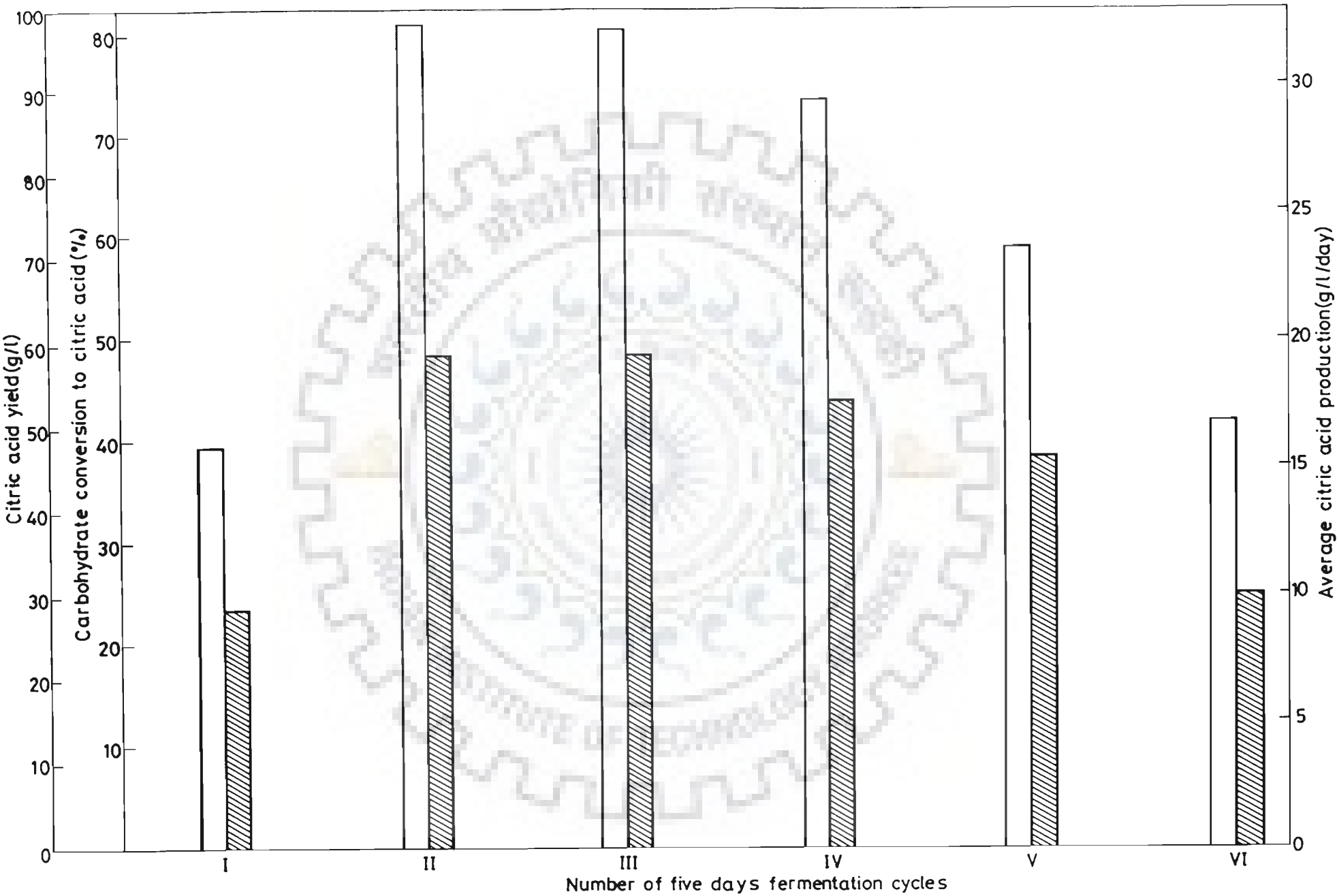

Citric acid yield(g/l)
Carbohydrate conversion to citric acid(%)
Average citric acid production(g/l/day)
Number of five days fermentation cycles
I
II
III
IV
V
VI

Fig.33

For this purpose, two types of immobilizing systems, namely calcium alginate beads (4mm diameter) and polyacrylamide gel slab pieces (4x4x1 mm) containing *A. niger* KCU520 cells, were employed and conditions for citric acid production from molasses were optimized. The results are described below:

4.5.1 Citric acid formation from molasses as a function of inoculum level

The inoculum level was found to have a profound effect on citric acid production, using immobilized cells, unlike free cell fermentation (Fig.34). In both systems, maximum citric acid yield was obtained at 20% inoculum level. Higher concentration of inoculum was found to be much inferior for citric acid production. Perhaps, a high concentration of inoculum hindered the aeration/agitation process and also the diffusion characteristics across the immobilized bed.

4.5.2 Citric acid formation as a function of temperature

The optimum temperature for citric acid fermentation performed with immobilized cells was found to be 32°C and 35°C for Ca-alginate and polyacrylamide immobilized *A. niger* KCU520 cells, respectively (Fig.35). This temperature is slightly higher than the optimum temperature (30°C) obtained with free cells.

4.5.3 Time course of citric acid production with immobilized cells

The time course for conversion of sugars to citric acid during fermentation using immobilized cells is shown in Fig.36 and 37. The results show that in the case of Ca-alginate immobilized cells, 12-14 days period is required for apparently complete fermentation with a maximum yield of 45 mg/ml citric acid, which corresponds to 45% conversion of molasses sugars to citric acid (Fig.36). Fermentation periods longer than 14 days did not result in any further increase in citric acid concentration in the broth. On the other hand, using PAG immobilized cells, it was found that citric acid accumulation increased sharply upto

FIG.34 CITRIC ACID FERMENTATION AS A FUNCTION OF IMMOBILIZED CELL INOCULUM.

Varying amount of calcium-alginate beads (4 mm diameter) and polyacrylamide slab gel (4x4x1 mm) containing immobilized cells of *A. niger* KCU520 were used to inoculate the fermentation medium as described under Methods. The immobilized cells were acclimatized for 5 days in fermentation medium under optimum conditions before using them for actual fermentation. For fermentation, calcium- alginate immobilized cells were inoculated in flasks in an orbital shaker at 150 rpm. Polyacrylamide gel immobilized cells were packed in a vertical column (20x5 cm) of a single- stage bioreactor. The immobilized inoculum level is represented by g/ml medium. Citric acid produced during fermentation was determined as described under Methods. (o——o) and (Δ—Δ) shows citric acid production by PAG and Ca- alginate immobilized cells of *A. niger* KCU520, respectively.

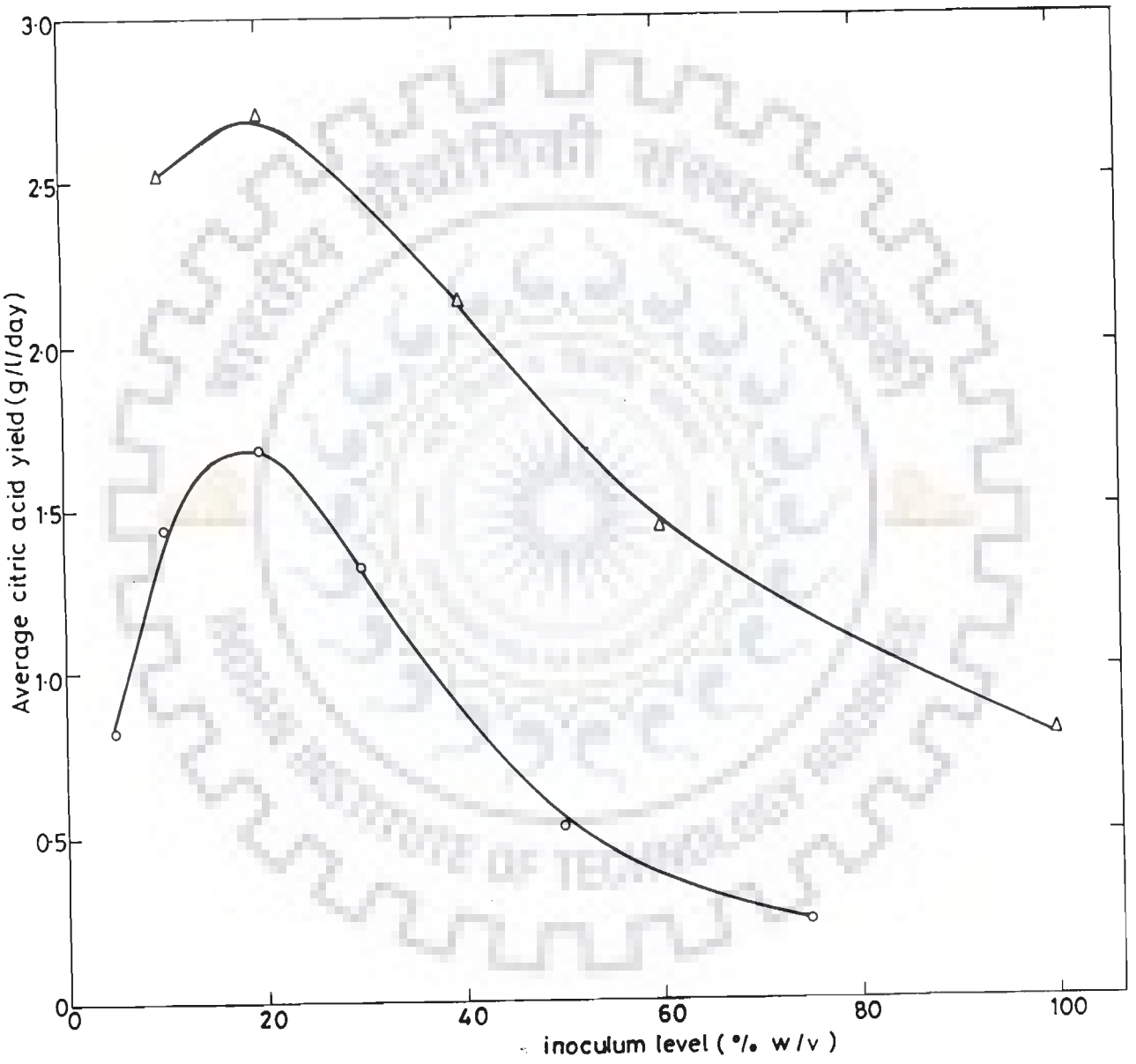

3·0
2·5
2·0
1·5
1·0
0·5
0
0
20
40
60
80
100
Average citric acid yield (g/l/day)
inoculum level (% w/v)

Fig. 34

FIG.35 EFFECT OF TEMPERATURE ON CITRIC ACID PRODUCTION BY IMMOBILIZED A. niger KCU520 CELLS.

Immobilized *A. niger* KCU520 cells in 4 mm diameter calcium- alginate beads or polyacrylamide slab gel (4x4x1 mm slice) were used for citric acid fermentation after 5 days of acclimatization in fermentation medium under optimum conditions. Fermentation with calcium-alginate immobilized cells was carried out in flasks in an orbital shaker at 150 rpm, while PAG immobilized cells were packed in a single- stage bioreactor for citric acid fermentation. Fermentation was carried out at indicated temperatures under optimum fermentation conditions. (o—o) and (Δ—Δ) shows an average citric acid production (g/l/day) by PAG and Ca-alginate immobilized cells, respectively.

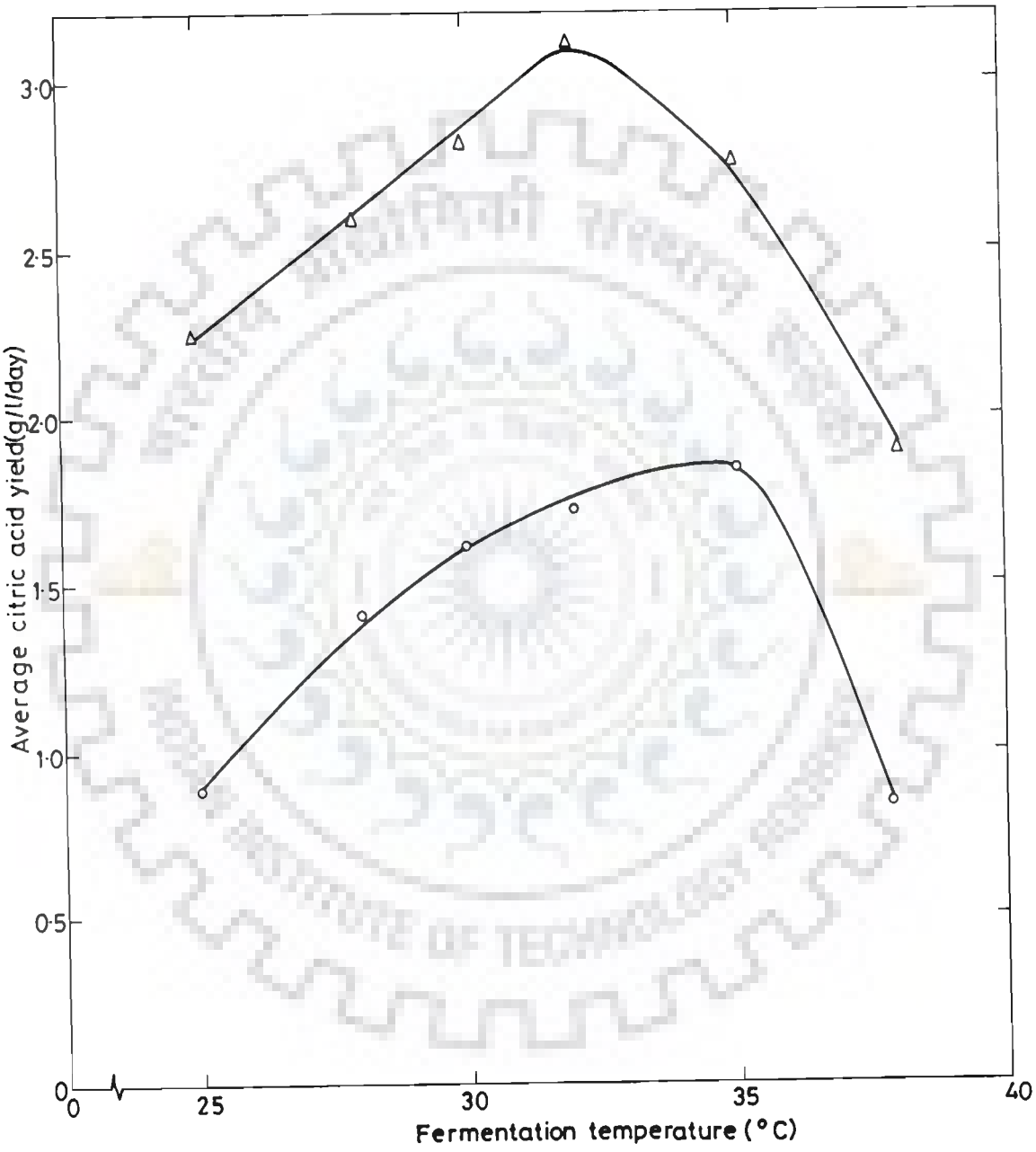

Average citric acid yield(g/l/day)
3·0
2·5
2·0
1·5
1·0
0·5
0
0
25
30
35
40
Fermentation temperature (°C)

Fig. 35

FIG.36 TIME COURSE OF CITRIC ACID FERMENTATION USING CALCIUM- ALGINATE IMMOBILIZED CELLS OF A. niger KCU520.

Fermentation was carried out with Ca-alginate beads containing immobilized *A. niger* KCU520 cells (10^7-10^8 spores/ml) in a flask at 32°C in an orbital shaker, shaking at 150 rpm, for the indicated periods. Clarified sugarcane molasses containing 10% sugars and adjusted to pH 4.0 was used as substrate. The amount of citric acid formed was determined by standard procedure described under Methods. Citric acid yield is represented as g/l medium.

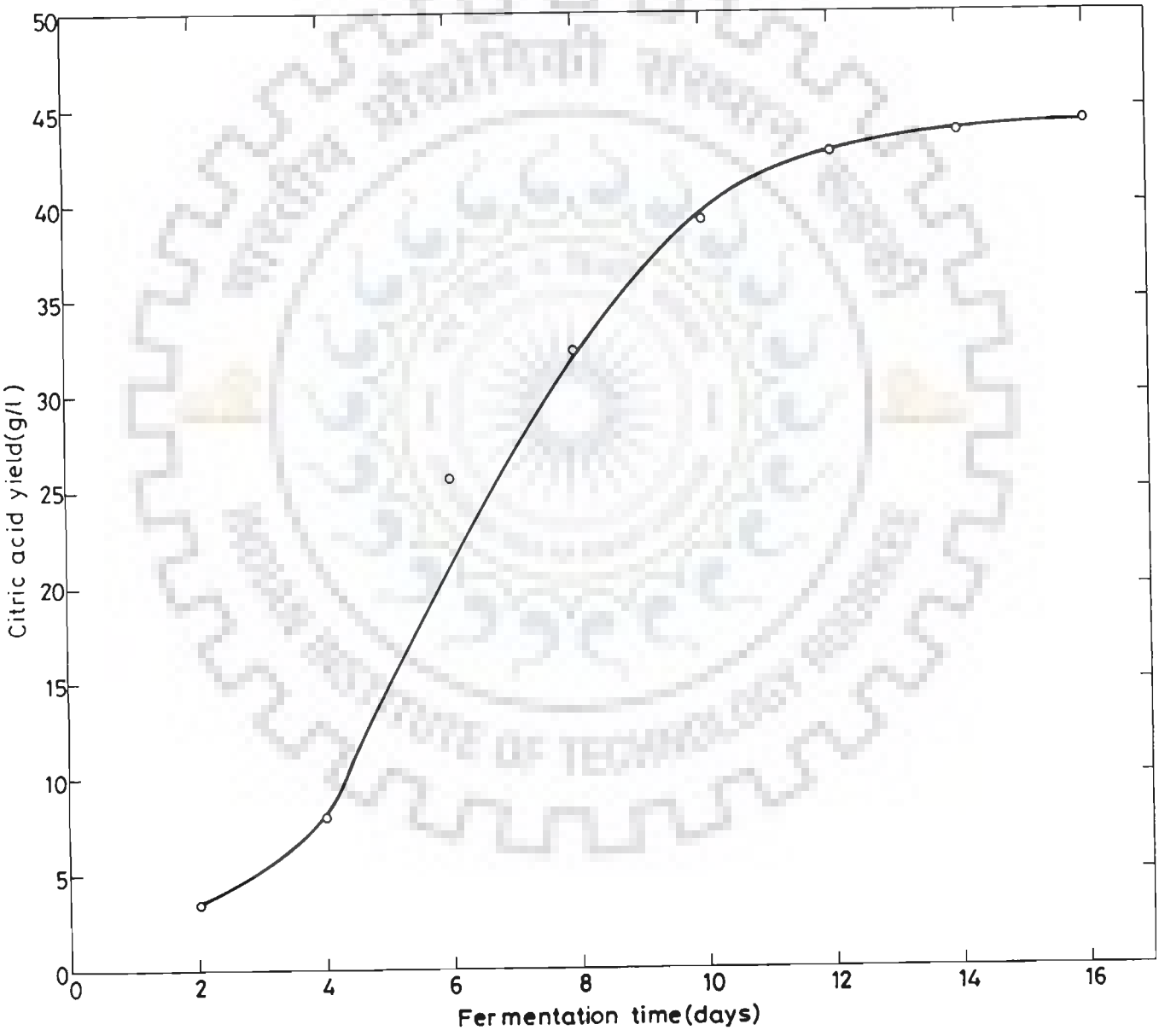

Citric acid yield(g/l)
50
45
40
35
30
25
20
15
10
5
0
0
2
4
6
8
10
12
14
16
Fermentation time(days)

Fig. 36

FIG.37 TIME COURSE OF CITRIC ACID FERMENTATION USING POLYACRYLAMIDE IMMOBILIZED CELLS.

Immobilized cells of *A. niger* KCU520 in 4x4x1 mm sliced polyacrylamide gel were used for citric acid production in a single-stage bioreactor, using 0.1% HCF-treated molasses containing 10% sugars. Multiple cycle fermentation was carried out under optimum fermentation conditions for 24 h (one cycle) after which the fermented broth was removed and fresh medium added for another cycle of fermentation. In this way 20 fermentation cycles were performed. After each cycle the amount of citric acid (g/l) was determined as described under Methods. Note the full viability of the immobilized cells upto 20 days and maximum citric acid yield 12 g/l/day from 6th to 20th-cycle.

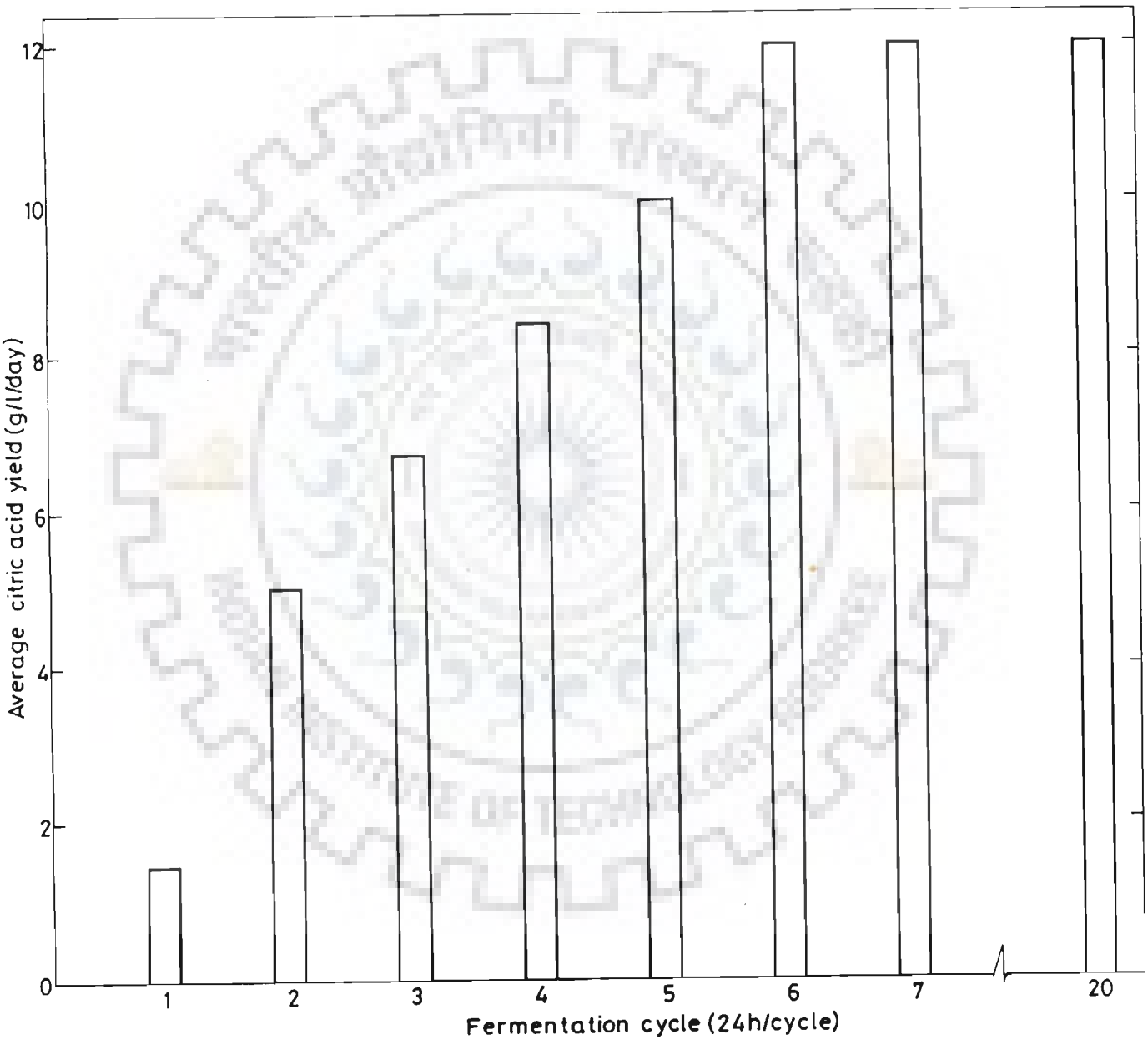

Average citric acid yield (g/l/day)
0
2
4
6
8
10
12
1
2
3
4
5
6
7
20
Fermentation cycle (24h/cycle)

Fig. 37

5 days of fermentation and thereafter it reached a steady state at which no further increase in citric acid production was observed even by increasing the incubation time (Fig.37). However, it can be seen that after a 5 days initial lag, the citric acid production increased rather sharply, indicating that the immobilized cells, like free cells, required 5 days for proper maturation and acclimatization for efficient conversion of sugars to citric acid under optimum conditions of temperature, pH, substrate concentration and aeration. In addition, the results suggest that the immobilized cells after proper acclimatization, may be used in subsequent fermentation cycles.

4.5.4 Use of immobilized cells for repeated fermentation cycles

From the results discussed in the preceding section, it is apparent, that the immobilized cells may be used again with fresh medium and that 5 days acclimatization of cells would give better yields. Experiments were conducted in which Ca-alginate immobilized cells were acclimatized for 6 days in fermentation medium under optimum conditions. The fermented broth was then replaced afresh and the daily yield of citric acid was monitored. The results are shown in Fig.38. It was found that 60% sugars were converted into citric acid during the second, third and fourth cycles, each requiring only 7 days. In other words, after acclimatization of the immobilized cells for 5-6 days, the same batch of immobilized cells was used in three fermentation cycles without any significant loss in productivity. These results are identical to that obtained for multiple cycle SSF (Fig.33), except that in the case of immobilized cells the yield of citric acid was 20% lower and fermentation time 2 days longer than SSF. Nevertheless, the use of immobilized cells for citric acid production seems promising. However, possibility of using Ca-alginate immobilized cells in continuous production of citric acid is doubtful because of the long period (7 days) required for fermentation; even after proper acclimatization of cells.

A similar approach was used for the polyacrylamide gel immobilized cells. In a typical experiment, the immobilized cells were acclimatized in the fermentation broth under optimum conditions for 1 to 8 days. The fermented broth was removed and fresh medium was added. After 24 h of fermentation, citric acid content was estimated. The results shown in Fig.39 represent citric acid fermentation data in single and two-stage bioreactors.

FIG.38 USE OF CALCIUM-ALGINATE IMMOBILIZED A. niger KCU520 CELLS FOR MULTIPLE CYCLES OF CITRIC ACID FERMENTATION IN A SINGLE-STAGE BIOREACTOR.

Calcium-alginate immobilized *A. niger* KCU520 cells were loosely packed in a single-stage bioreactor column (Fig.4) and fermentation was carried out under optimum fermentation conditions using clarified sugarcane molasses containing 10% sugars as substrate. The citric acid formation was monitored every 24 h. Note the maximum rate which is achieved in 7 days. Four successive 7 days fermentation cycles were performed. (☐) daily citric acid production (g/l) from 1st to 7th day ; and (▨), average citric acid production in 7 days (g/l).

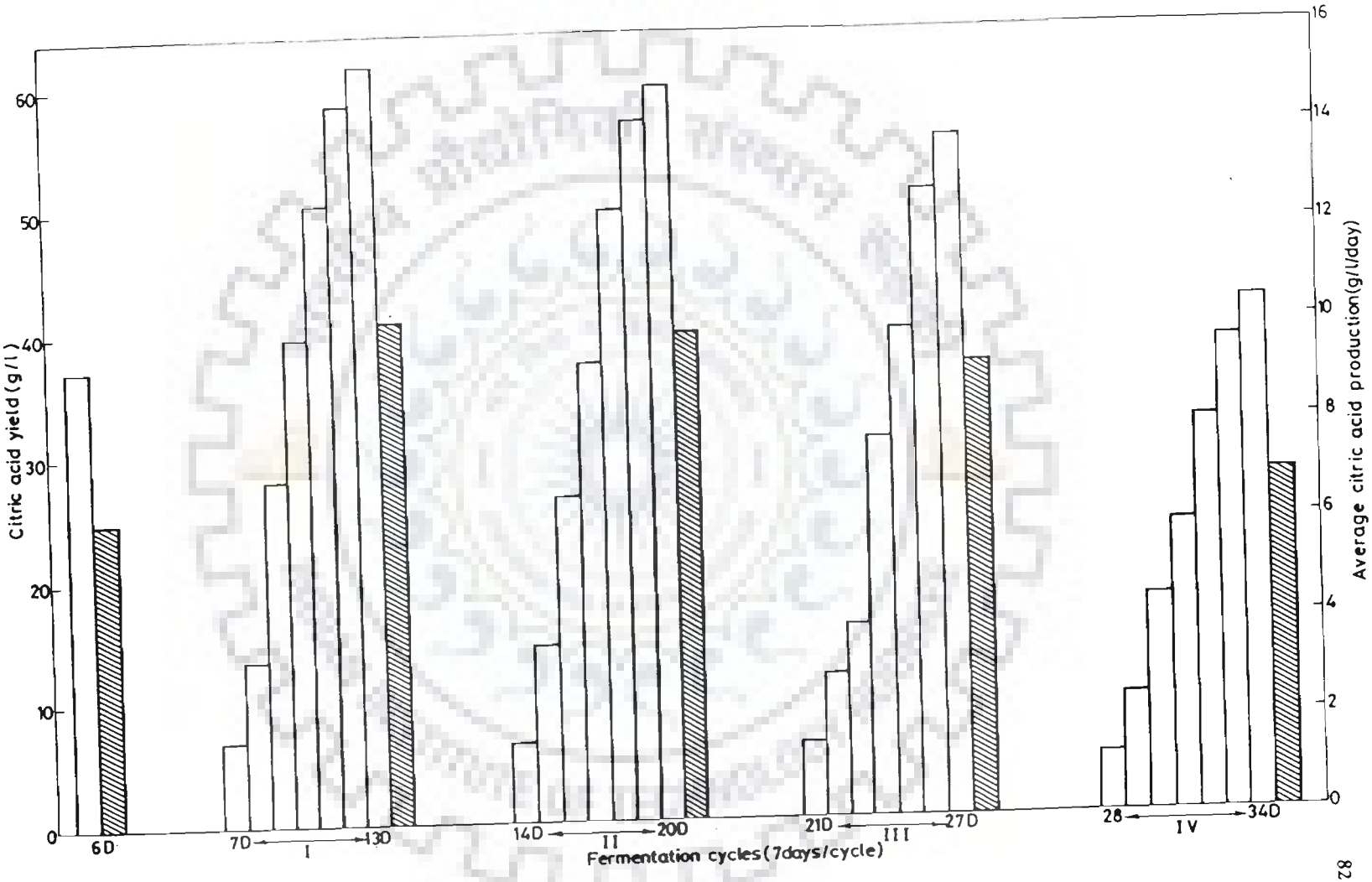

Citric acid yield (g/l)
Average citric acid production(g/l/day)
Fermentation cycles(7days/cycle)
6D
7D
13D
14D
20D
21D
27D
28
34D
I
II
III
IV

Fig. 38

FIG.39 CONTINUOUS CITRIC ACID PRODUCTION IN A TWO-STAGE BIOREACTOR USING POLYACRYLAMIDE GEL IMMOBILIZED CELLS.

Polyacrylamide immobilized cells of *A. niger* KCU520 were used for citric acid production in a two-stage bioreactor (Fig.5) under optimum fermentation conditions using clarified molasses (10% sugars) as substrate. 24 h fermentation cycles were carried out. After 24 h, the fermented broth from Ist- stage bioreactor column was transferred to the IInd-stage column for fermentation for another 24 h. Simultaneously, the Ist-column was filled with fresh fermentation medium. After 24 h fermentation in the IInd-column, the fermented broth was removed from the IInd-column followed by transfer of the fermented broth from the Ist-column to the IInd-column. The amount of citric acid in the fermented broth was determined as described under Methods. The results show the citric acid formation (g/l/day) in 20 successive fermentation cycles, in both Ist-stage and IInd-stage fermentation. (☐) and (▨) show amount of citric acid formed after Ist- and IInd-stage fermentations, respectively.

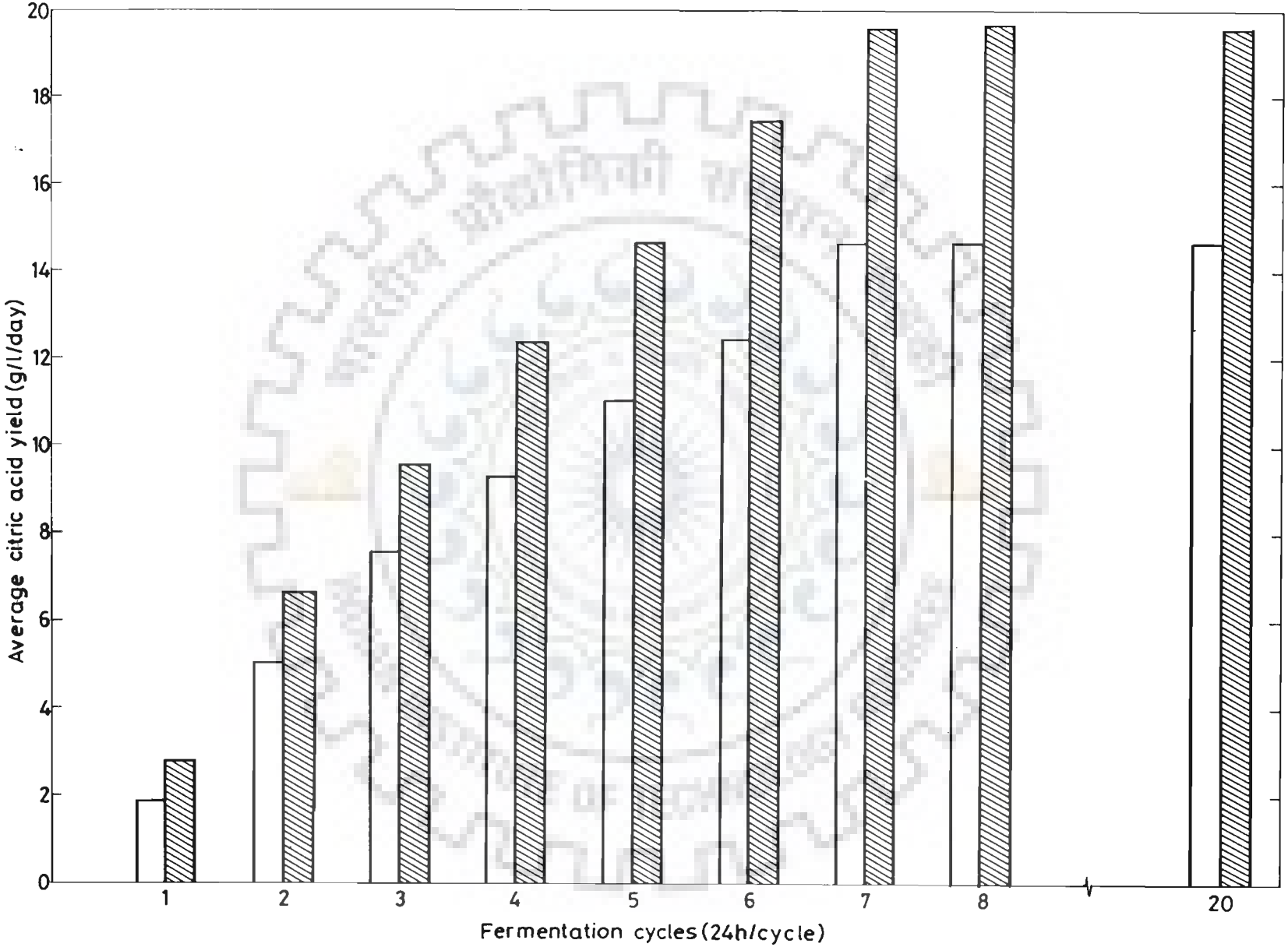

Average citric acid yield (g/l/day)
0
2
4
6
8
10
12
14
16
18
20
1
2
3
4
5
6
7
8
20
Fermentation cycles (24h/cycle)

Fig. 39

As expected, a two-stage fermenter gave a better yield. The important point is that the citric acid production increased with acclimatization period upto 6 days. Thereafter citric acid production was stabilized at 20 g/lit/day, corresponding to 20% conversion of sugars to citric acid. In addition, the immobilized system remains viable for 20-30 days. In other words, polyacrylamide gel immobilized cells once acclimatized for 6 days can be in continuous operation for production of citric acid in a two-stage fermenter with an efficiency of 20% conversion of sugars to citric acid per day for nearly 30 days. The system could be prolonged further, but due to some problems with experimental set-up, further incubation was not pursued. The scaling- up studies using this immobilized system are in progress.

4.6 EXTRACTION OF CITRIC ACID FROM FERMENTED BROTH

Citric acid extraction was done by the classical method of calcium hydroxide precipitation (Fig.6). A material balance of citric acid was monitored during the extraction and purification procedure (Fig.40). After separation of fungal mycelium (11-12 g/l dry wt.), fermented broth was subjected to citric acid extraction. Practically no loss in citric acid recovery was observed during calcination, acidulation and decolourization steps. The major loss (nearly 10%) in citric acid recovery occurred during the crystallization process, giving approximately 88% pure citric acid crystals. Thus, starting from 120 g/l sugars in molasses, about 80 g of pure citric acid was recovered from the fermented broth obtained after 10 days of SSF.

FIG.40 FLOW DIAGRAMME FOR THE RECOVERY OF CITRIC ACID.

The citric acid yield at each step of down stream process of the post fermentation operation is indicated. Starting with 120 g sugars 80 g of citric acid crystals were obtained.

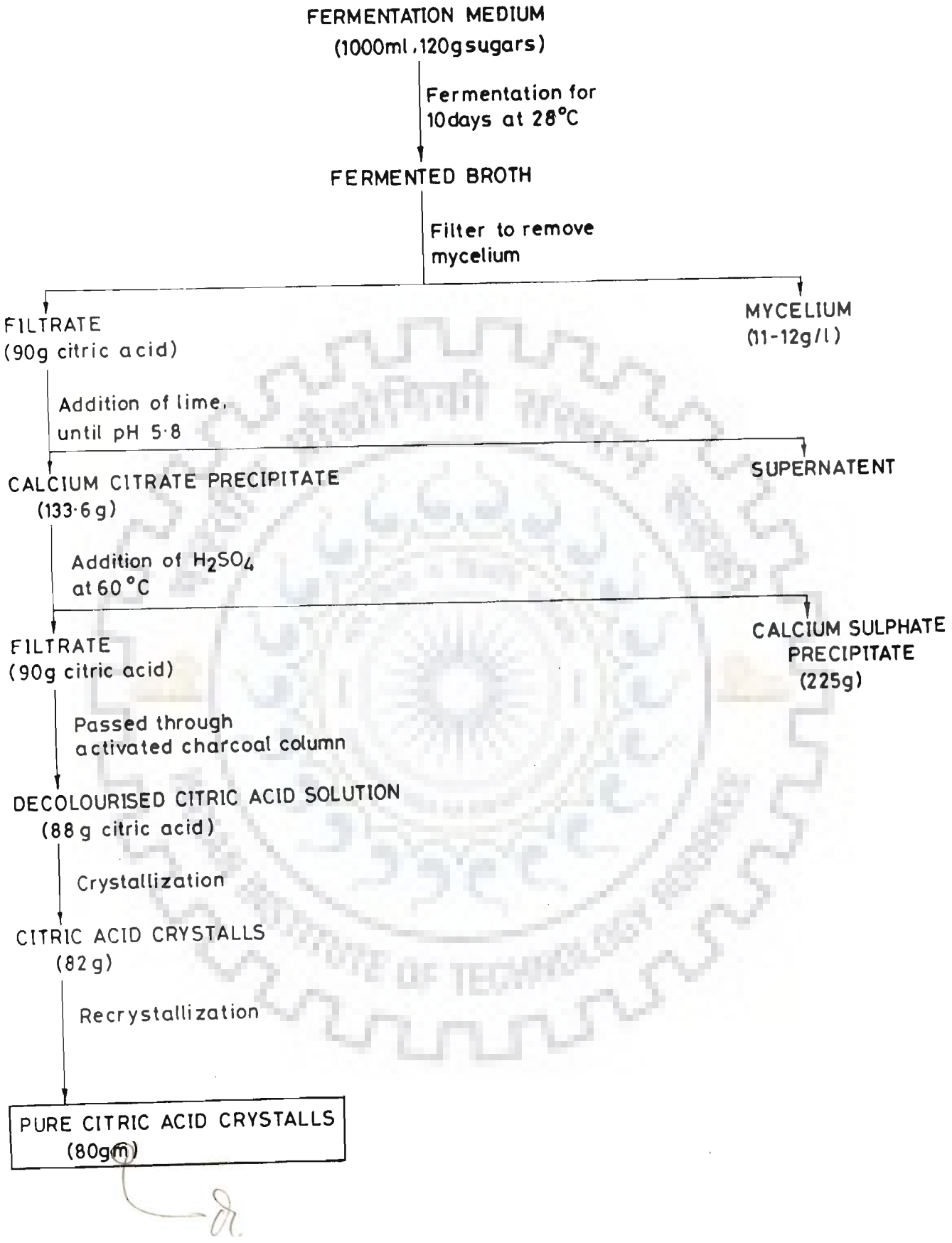

FERMENTATION MEDIUM
(1000ml, 120g sugars)
Fermentation for
10days at 28°C
FERMENTED BROTH
Filter to remove
mycelium
FILTRATE
(90g citric acid)
MYCELIUM
(11-12g/l)
Addition of lime,
until pH 5·8
CALCIUM CITRATE PRECIPITATE
(133·6g)
SUPERNATENT
Addition of H_2SO_4
at 60°C
FILTRATE
(90g citric acid)
CALCIUM SULPHATE
PRECIPITATE
(225g)
Passed through
activated charcoal column
DECOLOURISED CITRIC ACID SOLUTION
(88g citric acid)
Crystallization
CITRIC ACID CRYSTALLS
(82g)
Recrystallization
PURE CITRIC ACID CRYSTALLS
(80gm)

Fig. 40

5.0 DISCUSSION

5.1 IMPROVEMENT OF CITRIC ACID PRODUCING STRAIN OF *A. niger* BY UV MUTAGENESIS

For any successful fermentation process an efficient microbial strain is a prerequisite. From the work of various laboratories (Currie, 1917; Bernhauer, 1928; Foster, 1949; Johnson, 1954; Prescott and Dunn, 1959; Perlman and Sih, 1960; Berry et al., 1977; Rohr et al., 1983), it was obvious that citric acid fermentation is an extremely complex process that requires both an appropriate strain and precise fermentation conditions (Shu and Johnson, 1948a; Gardner et al., 1956; Trumpy and Millis, 1963; Choudhary and Pirt, 1966; Sanchez-Morroquin et al., 1970; Banik, 1975). So, the two main objectives of this work were, (i) to obtain an efficient high yielding microbial strain and (ii) to define precise optimal conditions for citric acid fermentation from sugarcane molasses.

It is generally agreed that only some selected strains of *Aspergillus niger* fungus are best suited for citric acid production (Perlman and Sih, 1960; Lockwood, 1975). Keeping this point in mind, various strains of *A. niger* were isolated from nature and screened for citric acid production. Among them, *A. niger* KCB1 was found to be the best, giving nearly 35% carbohydrate conversion to citric acid. But this yield was not enough for commercial production of citric acid. Hence, methods were sought to improve this parental strain.

It is believed that citric acid, as a product of primary metabolism is not likely to be excreted under natural conditions in noticeable amounts. Thus, any appreciable excretion must be regarded as the result of some severe irregularity of metabolism caused by genetic deficiencies or by drastic metabolic imbalances (Rohr et al., 1983; Kubicek and Rohr, 1986). Since the technique of induced mutation and strain selection has now been accepted as a routine practice to improve microbial strains for industrial production of citric acid (Perlman and Sih, 1960; Das, 1972; Berry et al., 1977; Rohr et al., 1983), UV radiations were used for induction of mutations. The parental strain *A. niger* KCB1 was exposed to UV rays for different time periods and then screened for citric acid productivity by calculating Acid Unitage (AU) values.

Storage of strains obtained through mutagenesis for longer period of time is known to show degeneration and loss of citric acid producing capacity (Johnston, 1974). Furthermore,

during fermentation the mutants tend to revert or degenerate (Perlman and Sih, 1960; Rohr et al., 1983). The screening method (Rohr et al., 1979) adopted in this work was fairly accurate, fast and convenient, ensuring rapid screening of mutants. By simply calculating AU values, the efficiency of each mutant was easily monitored without laborious and time consuming fermentation trials. That, the AU value was a true representation of citric acid producing capability of any strain, was further confirmed by the linear correlation obtained between AU values and citric acid produced in fermentation trials (Fig.8).

As expected, UV exposure of parent *A. niger* cells yielded both plus and minus mutants. In the Ist-step mutagenesis, the proportion of minus mutants was low as compared to the IInd-step mutagenesis (Fig. 9 and 10). This was probably due to the greater stability of the parental strain (isolated from nature) used in Ist-step than the mutated strain, *A. niger* KCU5, which was used in the IInd-step mutation. The AU value of most of the reversion of *A. niger* KCU5, that occurred, was in the region of parental strain (Fig.9).

No direct correlation between UV exposure and % plus mutants could be worked out, since mutations by UV are highly nonspecific. However, it was obvious that chances of getting the desired mutant were more with longer UV exposures than that from short exposures. It may be pointed out, however, that since the UV exposure method is a 'hit and trial' method, reproducibility of results is a remote possibility. Fortunately, *A. niger* strain KCU520, obtained after two-stage mutagenesis, was found to be stable for over several years with regard to its morphology and ability to convert sugars into citric acid.

Morphological characterization of *A. niger* KCU520 strain was done in order to correlate the change in the mycelium structure after mutagenesis with the citric acid producing capability of the mutant. Interestingly, morphological characters of *A. niger* KCU520 are related to its high citric acid yielding capability. From the appearance itself, the two fungi seem to be very different. For instance, *A. niger* KCB1 mycelium was yellowish in colour with an abundance of spores on it, while in contrast *A. niger* KCU520 mycelial mat was white in colour with relatively lesser number of spores on it (Fig.11). Scanning electron microscopic studies revealed that *A. niger* KCB1 mycelium was thin, elongated and less branched while the *A. niger* KCU520 mycelium was thick, short and contained various unusually globular type of cells in between and at the tip of mycelium (Fig.12).

The yellowish colour of mycelium is an indication of a faulty fermentation and is due to

the formation of the pigment 'asperenone' (Jefferson, 1967). The formation of asperenone is believed to occur in the presence of excess of trace metals (Rohr et al., 1983). This indicates that *A. niger* KCU520 is a more metal resistant strain since its mat colour remains white during fermentation than its counterpart *A. niger* KCB1, which becomes yellow, under similar fermentation conditions. The low spore population also appears to be related to high citric acid yield as Lockwood (1975) has observed that sporulating mycelium contributes less to the citric acid synthesis due to the pigment formation during sporulation.

The main aim of this study was to produce citric acid from sugarcane molasses, which contains substantial amount of trace metal ions (Fe^{3+}, Cu^{2+}, Zn^{2+} and Mn^{2+}), which are extremely inhibitory for citric acid formation from sugars. Thus, the high metal resistance of *A. niger* KCU520 is an important characteristic which may be particularly useful from the industrial point of view .

5.2 OPTIMIZATION OF CITRIC ACID FERMENTATION BY *A. niger* KCU520:

As stated earlier, precise optimum conditions are essential for citric acid fermentation (Shu and Johnson, 1948a; Prescott and Dunn, 1959; Gupta et al., 1976; Berry et al., 1977; Rohr et al., 1983; Xu et al., 1989). This should be more relevant in the case of new strain and the type of molasses used. The composition of molasses varies from region to region and also from one variety of sugarcane to another. The molasses used in this study was from a typical sugar factory in Western Uttar Pradesh (India) which uses a mixture of varieties of sugarcane. Thus, the composition of molasses with respect to trace metals, nitrogen, phosphorus and sugars was not entirely consistent. Analysis of molasses at different times over the four years supported this view.

The results presented here indicate that the nature of the sugar source exhorts a strong effect on citric acid production. Sucrose, undoubtedly was found to be the most favourable substrate followed by glucose, glycerol and fructose. Galactose gave a very poor yield (Fig.13). Monosaccharides like glucose and fructose should be readily metabolized by fungus and expected to give at least comparable yields of citric acid as with sucrose. But this is not the case. In some instances fructose and equimolar mixture of glucose and

fructose, have been reported as decidedly inferior to sucrose (Karow, 1942). These results obtained were in agreement with those reported by other workers (Bernhauer, 1928; Prescott and Dunn, 1959; Naguchi, 1962; Hossain et al., 1984).

Hossain et al. (1984) related this phenomenon to the activity of the key regulatory enzymes. They found that 2-oxoglutarate dehydrogenase was a key regulatory enzyme for citric acid production and showed that by suppressing its activity, high citric acid yields could be achieved. Further, low citric acid production from galactose was attributed to the high activity of 2-oxoglutarate dehydrogenase enzyme. In addition, aconitase activity is also known to have a direct correlation with citric acid accumulation as the enzyme activity was highest when sucrose was the sugar source and lowest when galactose was used (Hossain et al., 1984).

Sugars are used for the growth of the organism as well as for product formation. At a low sugar concentration, most of the substrate is consumed by the fungus for its growth and only a little substrate is left for citric acid production. On the other hand, in the presence of very high concentration (about 15%) of sugars, the citric acid production declines because the mycelial growth increases (Fig.14). It is known since long that the conversion of sugars to citric acid takes place within the cell of *A. niger*. While, sugars enter the cell by osmosis, citric acid is known to diffuse out (Prescott and Dunn, 1959). Thus, at high sugar concentrations, the sugar uptake will be low due to a slow osmotic rate and side by side the diffusion of citric acid may also be hindered due to a concentration gradient across the mycelial mat. In addition, due to the presence of substances that inhibit growth of *A. niger* (Shu and Johnson, 1948b; Chaudhary et al., 1972) and also the process of spore germination (Fencl and Leopold, 1957; 59), high concentration of molasses may indirectly affect the citric acid production. The optimum sugar concentration for *A. niger* KCU520 was found to be about 12%. At this concentration of sugar, the critical concentration of biomass that is necessary for efficient conversion of sugar to citric acid is produced. In this respect, the pattern of citric acid fermentation using *A. niger* KCU520 resembles that observed by other workers, (Currie, 1917; Doelger and Prescott, 1934; Xu et al., 1989) who have used other strains of *A. niger*.

Sussman and Halvorson (1966) were of the opinion that spore age has a distinct effect on spore viability, while Chaudhary et al. (1978) stated that spores from 3 days old slants were as good as spores from 7-8 days old culture, for citric acid production. Thus, there is no

common opinion regarding the age related viability in spores of *A. niger* when used for citric acid fermentation. In our experiments, we observed that spore viability for citric acid accumulation varied with the age of spores. There seems to be a critical period in which spores are most active for citric acid production and this critical age of spores may be different for different strains. The parental strain *A. niger* KCB1 was found to have a wide active spore age range (3-5 days), while for *A. niger* KCU520 the active spore age was 5-days (Fig.15). The biomass pattern also changes with spore age, showing that spore age has some effect on the germination and growth properties of the organism.

The importance of incubation temperature in determining the yields of citric acid has been emphasized previously (Kovats, 1946; Tomlinson et al., 1951; Kitos et al., 1953). Our results also show a profound effect of temperature on the citric acid fermentation. The optimum temperature (26-28^{o}C) found for *A. niger* KCU520 was in the general range used for other citric acid producing strains of *A. niger* (Doelger and Prescott, 1934; Kitos et al., 1953). At this temperature there was a proper balance in biomass production and rate of citric acid formation from carbohydrates.

Kitos et al. (1953) correlated the influence of temperature with mineral requirement (Cu, Zn, Fe and Mn) of fungus for citric acid production. They showed that at low temperature, the trace metal resistance of fungus was more as compared to higher temperature. In this respect, it is relevant to highlight that the *A. niger* KCU520 had a lower temperature optima for citric acid production than the parental strain *A. niger* KCB1 (Fig.16).

The modern concept of citric acid fermentation process stems from the outstanding discovery of Currie (1917) that *A. niger* was capable of growing abundantly at an initial pH of 2.5 to 3.5 and would accumulate high concentration of citric acid in the medium if the pH of the growth medium was below 2.0. However, the picture is entirely different when molasses was used for citric acid production where fermentation is generally carried out at higher pH due to the presence of various growth inhibitory substances present in molasses (cf. Perlman and Sih, 1960; Rohr et al., 1983). The *A. niger* KCU520 strain, which was used in this study, was found to have two pH optima, pH 1.75 and pH 4.0 (Fig.17). The pH 4.0 was more favourable when molasses was used as the carbon source whereas pH 1.75 was preferred when pure sucrose was the substrate. In fact, with molasses as carbon source, the spores of *A. niger* KCU520 failed to germinate when the pH of the fermentation medium was below pH 3.5. Thus, the maintenance of optimum pH is extremely important

for the successful progress of fermentation. During the course of fermentation, the pH of the medium gradually drops. This might be useful in curtailing the problems arising from contamination since few microbes can develop in conditions of high acidity.

A typical curve of growth and production was obtained during citric acid fermentation, showing lag, log, retardation and stationary phases (Fig.19). The initial two days of fermentation show negligible fungal growth and low citric acid production, depicting a lag phase in which spores germinate and grow in the form of mycelium. As growth reaches in the log phase, the acid yield also increases exponentially. After nearly 10 days, the retardation phase begins to set in and fermentation is completed in 12-14 days depending upon the strain. The results obtained are in full agreement with the work of others, cited in literature (Gaden, 1955; Chmiel, 1975a; 75b; Verhoff and Spradlin, 1976; Rohr et al., 1981).

According to the classical scheme of Gaden (1955), citric acid fermentation by *A. niger* is considered a type II fermentation. This type of fermentation is characterized by the fact that there are two rate maxima with regard to growth and product formation. In the first phase of fermentation,growth is accompanied by minimal product formation, whereas in the second phase, product formation reaches a maximum but growth rate is low. The results obtained in the present study are generally consistent with this pattern except that an increase in biomass precedes the second production phase as well, implying that the second production phase is also growth associated (Fig.20).

In the retardation phase of fermentation, the decline in the citric acid production after 10-12 days of fermentation was probably due to the lysis of active *Aspergillus niger* cells, change in optimum pH, accumulation of citric acid or formation of deeply packed monolayers of the cells which fail to regenerate (Prescott and Dunn, 1959; Berry et al., 1977). In addition, it is of common knowledge that as the fungal cells grow older, the cytoplasmic contents also start reducing, showing a decline in activity due to aging of cells.

Accumulation of large amounts of citric acid is the result of metabolic malfunction of *A. niger* in a medium of low pH value with a high sugar content and carefully controlled concentration of other nutrients (Foster, 1949; Prescott and Dunn, 1959; Perlman and Sih, 1960; Kapoor et al., 1982; Rohr et al., 1983; Kubicek and Rohr, 1986). Citric acid production by *A. niger* involves both, a 'growth stage' and a 'fermentation stage'. The

organism, therefore, needs major elements such as carbon, nitrogen, phosphorus, magnesium, etc., in addition to various trace elements (Von Loesecke, 1945; Shu and Johnson, 1948a; Martin and Steel, 1955; Trumpy and Millis, 1963), for growth and citric acid production. Further, it may be assumed that since carbon-dioxide is the normal end product of carbohydrate utilization, conditions under which citric acid accumulates are abnormal and may be considered to be the result of nutritive deficiencies in the medium. One of the nutritional conditions for the optimum production of citric acid from sugars is a definite N:P ratio in the fermentation medium (Perquin, 1938; Johnson, 1954; Trumpy and Millis, 1963). For instance, if N:P is less, i.e., phosphate is in excess in the medium, it will promote growth and more substrate will be consumed to produce biomass, resulting in low citric acid production (Khan et al., 1970). On the other hand if N:P is too high, then also citric acid production is low, since higher nitrogen content leads to the accumulation of oxalic acid (Naguchi and Bando, 1960; Gupta et al., 1976). Therefore, in order to achieve an abundant excretion of citric acid, growth of the organism must be restricted. The precondition for sufficient citric acid production is a medium deficient in one or more major nutrients like P, so that growth of the fungus can be checked and more substrate can be utilized for citric acid production. In an abundance of nutrients, mould will oxidize sugar completely to CO_2 and H_2O (Johnson, 1954). Therefore, it is probable that citric acid accumulation occurs as a result of an enzymic deficiency caused by the use of a medium deficient in one or more essential nutrients, i.e., P.

5.3 EFFECT OF SOME REGULATORS ON THE CITRIC ACID FERMENTATION BY *A. niger* MUTANT KCU520

In disagreement to the enhancement of citric acid fermentation by the addition of vegetable oils, starch or H_2O_2 (cf. Rohr et al., 1983), the citric acid production by *A. niger* KCU520 was only marginally (10- 15%) increased. However, maintenance of a proper air supply throughout the fermentation process was found to be essential for obtaining high yields of citric acid.

There may be various effects of lipids for increasing citric acid yield (Millis et al., 1963). Lipids can alter the surface tension of the medium and thus improve the aeration efficiency in the case of surface fermentation. It is also possible that lipid may provide additional acetyl-CoA through beta-oxidation, for citric acid production. The improved citric acid

yield in the presence of lipid can also be on account of improved sugar utilization. The other widely applicable theory for lipid action on citric acid fermentation is that unsaturated fatty acids may also serve as alternate hydrogen acceptors during citric acid fermentation, thus allowing the mould to metabolize actively for longer periods of time, thereby improving the citric acid yield (Millis et al., 1963). Our results are also in agreement with this theory as it was observed that by addition of lipid the cells were found to be fairly active, to produce citric acid, for a longer period (Fig.25). Moreover, lipids were also found to stimulate the growth of fungus (Fig.21). The stimulation of growth is in agreement with the fact that these compounds are normally metabolized by *A. niger* and can function as carbon source.

Small amounts (0.1%) of H_2O_2 in the fermentation medium also enhanced the citric acid yield appreciably, while higher concentrations were found to be inhibitory (Fig.23). Earlier explanation to this effect was given as inhibition of aconitase hydratase enzyme by H_2O_2 (Bruchmann, 1961). But later this explanation was found to be incorrect as the aconitase hydratase was active during fermentation (Kubicek and Rohr, 1980). An alternative explanation may be that H_2O_2 may provide more dissolved oxygen to the medium, a favourable condition for citric acid accumulation (Shu, 1953; Clark and Lentz, 1961; Batti, 1966; Kovats and Sackowska, 1976; Kubicek et al., 1980). This may account for the high citric acid production observed in presence of H_2O_2. Support to this contention comes from the fact that in the present study H_2O_2 was added before sterilization which ensured its break-down thereby increasing the dissolved O_2 in the medium.

The time course of citric acid accumulation in the presence of H_2O_2 also strengthened the dissolved oxygen theory. It may be noted that in both fermentation systems, the initial rate of citric acid production was much higher as compared to the control (Fig.25), since the dissolved oxygen (dO_2) of the medium was high due to breakdown of H_2O_2. But since the H_2O_2 addition was so small, the dO_2 of medium would be exhausted soon. This explains a high citric acid production rate in the early stages of fermentation which then slows down after some time indicating exhaustion of extra dO_2, provided by H_2O_2 to the medium. Further, it is well known that more oxygen supply in early stages of fermentation stimulates the citric acid accumulation (Clark and Lentz, 1961; Batti, 1966; Wendel, 1967).

The retardation of fungal growth by H_2O_2 at higher concentration (1% or above) might be

due to the conversion of sugars to sugar acids. These sugar acids not only lower the pH of the medium but also are inhibitory to citric acid fermentation (Martin and Steel, 1955). It was observed that when starch was added, the number of spores produced over mycelium were reduced to a significant extent in surface culture, which is a favourable condition for citric acid excretion. It is unlikely that the stimulation of citric acid production by starch is due to its utilization as carbon source, since it is a complex polysaccharide. Therefore, its hydrolysis by the organism is likely to be a rate limiting step for the sugar transport. Probably, starch is employed as an adjunct (Cimerman et al., 1976; cf. Rohr et al., 1983), which reduces the spore amount and also fermentation time, thus improving citric acid fermentation.

The importance of air for citric acid fermentation is well known and a large body of data is available in the literature (Shu, 1953; Steel et al., 1954; Steel et al., 1955; Martin, 1957; Clark and Lentz, 1961; Batti, 1966; Kovats and Sackowska, 1976; Kubicek and Rohr, 1980). Our results also confirm the need of air for citric acid accumulation. Nearly 100% increase in the citric acid production was observed when high aeration was facilitated to the fermentation system, as compared to non-aerated conditions, in both surface and submerged fermentation processes (Table-2).

The possible role of O_2 can be explained by the fact that O_2 is used as one of the substrate which is metabolically needed for the reoxidation of glycolytic NADH during citric acid fermentation (Kubicek and Rohr, 1980). Further, instead of a standard respiratory chain, *A. niger* also has an alternate SHAM (Salicyl-hydroxamic acid) sensitive branch. It is, therefore, suggested that a high oxygen tension is needed for maintaining the activity of SHAM-sensitive alternate respiratory chain (Kubicek and Rohr, 1980).

5.4 USE OF SUGARCANE MOLASSES AS SUBSTRATE FOR CITRIC ACID FERMENTATION BY *A. niger* KCU520

The main objective of the present study was to utilize sugarcane molasses for citric acid fermentation, since it is abundantly available and contains approximately 50% sugars. However, crude sugarcane molasses is a complex system and cannot be used as such for citric acid fermentation. Even after proper adjustment of sugar content (12%) and pH (4.5) of molasses, the yield of citric acid was poor (Fig.26). One of the main reason was the

presence of large excess of heavy metal ions, especially Fe^{3+}, Cu^{2+}, Zn^{2+} and Mn^{2+}, which strongly inhibit the citric acid fermentation by *A. niger*. In agreement with literature (Martin, 1955; Clark et al., 1965; Horitsu and Clark, 1966; Leopold and Valtr, 1969), treatment of molasses with appropriate concentration (0.1%) of HCF at 70-90°C for 15 min precipitated most of the heavy metals which were easily removed by filtration (Table-4). This clarified molasses proved to be a very suitable substrate for citric acid fermentation with *A. niger* mutant KCU520, as it gave comparable yield of citric acid with that of pure sucrose as substrate (Fig.26). The process of HCF treatment of molasses is relatively cheap and can be adopted industrially.

Besides removing heavy metal ions by forming insoluble metal hexacyanoferrate complex salts, HCF seems to retard the growth of *A. niger* when present above the optimum level of 0.1% in the fermentation medium. But this inhibitory effect of HCF was overcome by the addition of appropriate amounts of phosphate (Fig.28). Thus, by adjusting the HCF and phosphate contents of the medium, the metal ions can be efficiently precipitated and fungal growth can be maintained in such a way so as to give maximum citric acid yield.

A comparison of submerged, surface and solid state surface fermentation (SSF) processes for citric acid production by *A. niger* KCU520 showed that SSF was a method of choice in which bagasse was used as a solid material to absorb molasses and to provide the solid surface for the fungal growth (Fig.29). The possible reasons for better fermentation efficiency are as follows:

i) the bagasse soaked with molasses provided an extra large surface for the organism to grow,

ii) adequate supply of air for the growth and citric acid production was facilitated, and

iii) bagasse absorb various impurities of molasses, thus making SSF least susceptible to the molasses impurities.

The depth of the fermentation medium containing solid matrix, bagasse in this case, is an important parameter which was found to have profound effect on the yield and efficiency of citric acid production (cf. Foster, 1949; Perlman and Sih, 1960). As pointed-out earlier, the sugar conversion to citric acid brought about by intracellular enzymes, takes place

within the living cells that make-up the mycelium mat. Sugar passes by osmosis while citric acid diffuses out of the cells (Prescott and Dunn, 1959). Thus, it would be expected that in a deep vessel containing a large volume of medium, the progress of acid formation will be slower than that of large shallow pans in which a large surface area of mycelium is exposed to a relatively shallow layer of medium (Fig.31). One of the interesting aspect of the SSF was the possibility of reusing the same biomass, developed in the first fermentation cycle, in subsequent fermentation cycles. Our results showed that by replacing the fermented broth with the fresh medium, the average rate of citric acid production was greatly increased (Fig.33). In fact, the second fermentation cycle was completed in 5-6 days which was about half the normal fermentation period. In addition, the *A. niger* KCU520 cells remained active for nearly 20 days. In other words, at least four fermentation cycles could be performed without any significant loss of efficiency (Fig.33). After three weeks time, however, as the fungal mat starts getting older, the citric acid producing ability also decreases. The decline in citric acid production was probably due to lysis of active *A. niger* cells, accumulation of citric acid or formation of deeply packed monolayers of the fungal cells which fail to regenerate. In addition, during aging, the cytoplasmic contents of fungus also start decreasing, thus reducing the cell activity.

It is suggested that SSF replacement batch system can be of great importance for citric acid production since nearly twice the production can be achieved in the same period. Further, since the same mycelial mat is used repeatedly, operation for charging, cleaning and filling-up of fermenter will be greatly reduced and a semi- continuous process can be operated for nearly 20-25 days. This will cut down the labour and material input, thus reducing the cost of production. On the other hand, although this process seems to be very useful for citric acid production, but if applied on an industrial scale, various difficulties might be encountered. First, the technical feasibility of this process remains to be ascertained. The filling-up and emptying of the medium in surface culture without disturbing the culture may prove to be difficult and extra care has to be taken in order to prevent any damage to the mat. In addition, contamination problem may arise by prolonging the process. However, these problems can be resolved by proper designing of the bioreactor. Thus, it would be worthwhile to determine the technical feasibility of multiple cycle SSF.

5.5 PRODUCTION OF CITRIC ACID BY IMMOBILIZED WHOLE CELLS OF *A. niger* KCU520

In recent years, immobilized biocatalyst technology is gaining popularity in industrial fermentation and efforts are being made to produce various commercially important products by fermentation of immobilized whole cells. We have also investigated production of citric acid by immobilized whole cells of *A. niger* KCU520. A slight change in the optimum fermentation conditions of pH, temperature and substrate concentration was observed. The most significant change was the higher optimum temperature (32-35°C) than the free cell (28-30°C) (Fig.35).It was not unexpected since immobilization is known to bring about certain physical and physiological stress on the cell (Eikmeier et al., 1984; Tsay and To, 1987).

The citric acid formation with immobilized cells was significantly lower than free cell fermentation. The lowering of citric acid yield upon immobilization might be due to the various physical and environmental stress conditions prevailing, which prevent the cells from interacting freely with the environment. On the other hand, in free cell culture fermentation the cells are in direct contact with the medium, resulting in higher citric acid production than the immobilized system. It is already known that the half life of cells is extended upon immobilization, as growth of these cells is greatly retarded due to environmental stress, so that these cells can be maintained on minimum requirement for a longer period (Linko, 1980; Jack and Zajik, 1977; Horitsu et al., 1985).

The total citric acid production in alginate system was found to be higher as compared to PAG system. This might be due to the high concentration of PAG (18%) for cell immobilization, which makes cells very difficult to proliferate through matrix, thus representing a true immobilized cell system. On the other hand, due to probably large pore size of the alginate matrix, the immobilized cells were interacting more freely with the environment, i.e., diffusion of substrate and air, thereby increasing citric acid production. In addition, a large number of free cell population was observed when alginate immobilized cells were used. Probably alginate being a polysaccharide is utilized by the fungus in prolonged fermentation, thus releasing the cells from the matrix into the medium. This implies that the alginate system represents two types of fermentation, i.e., immobilized cell fermentation and also a free cell fermentation. On the other hand PAG system was found to be a true representation of immobilized whole cell (IWC) system. This might be the

reason for the difference in citric acid production observed between PAG and alginate systems.

The immobilized cells took nearly 5-6 days for acclimatization before reaching a steady state of maximum citric acid production. Probably during acclimatization these immobilized cells are activated and properly oriented to face the changed environment from free to restricted interaction with the medium (Fig.36 & 37). Although the citric acid yield was low, the immobilized cells remained fully active for a longer period than the free cells. This is a useful property which can be explored for a continuous production of citric acid in a single-stage or a multi-stage bioreactor. In view of the above, immobilized cells were packed in a vertical column bioreactor and fermentation was carried out in a batch process. The alginate- immobilized cells in the bioreactor were found to be unsuitable for the continuous production of citric acid as the amount of free cells increased with time, behaving like a multiple cycle SSF system with low yields (Fig.38). This was not pursued further.

The PAG immobilized cells were found promising as little free cell formation was observed even after 20 days fermentation period. Furthermore, after 6 days incubation under appropriate conditions, the citric acid production was although low but steady (Fig.37). Nevertheless, PAG-immobilized *A. niger* KCU520 were packed in a two- stage bioreactor. These cells were activated for about 6-7 days under optimum fermentation conditions. After that, in batchwise operation, nearly 20% carbohydrate conversion to citric acid was achieved in 24 h. A semi-continuous process was achieved by replacing the fermented broth after every 24 h fermentation. This process was continued upto 20 days without any significant loss in productivity. The process can be prolonged, but due to some technical problems in the experimental set-up, the fermentation was stopped after 20 days (Fig.39). Similar results were obtained by previous workers, working with immobilized cells (Eikmeier et al., 1984; Eikmeier and Rehm, 1984; Horitsu et al., 1985; Tsay and To, 1987). Thus, there is great potential for immobilized whole cells to be used in bioreactors for citric acid production. Further research will, however, be required to develop it into a commercial reality.

6.0 SUMMARY

In an attempt to obtain a high citric acid yielding mutant, a local wild type strain of *A. niger* (*A. niger* KCB1) was isolated and subjected to ultra-violet mutagenesis. As a result of two-step mutagenesis, a high citric acid yielding strain of *Aspergillus niger* (*A. niger* KCU520) was isolated. The new mutant gave nearly two fold higher 'Acid Unitage Value', higher rates of fermentation and much greater tolerance to heavy metal toxicity compared to parental strain. In addition, the electron micrographs of the mutant cells showed a markedly changed morphology, as the mutant cells were white in colour and thick, short and swollen with rough cell surface compared to the yellowish coloured and thin, thread like smooth structure of the parent strain.

The fermentation conditions for citric acid production by mutant *A. niger* KCU520 were optimized with sucrose as the substrate, since these conditions were used as standard for the comparison of yield when sugarcane molasses was used. Maximum citric acid yield was achieved in batch surface fermentation when sucrose concentration was 12-14%, and fermentation was carried out at pH 1.75 and 28° under high rates of aeration (2 l/min) for 12 days using 5 day old spores for inoculation. Under these conditions 80-85% sugars were converted into citric acid by fermentation with *A. niger* KCU520 compared to only 35-40% conversion of sugars into citric acid by wild type *A. niger* KCB1. The effect of various regulators, such as hydrogen peroxide, vegetable oils, starch and air, on citric acid fermentation was also studied in detail. It was observed that 0.05-0.1% H_2O_2, 2% groundnut oil or 0.3% starch in the fermentation medium enhanced the citric acid yield by 10-15% in both surface and submerged fermentation systems.

The optimum conditions thus obtained with sucrose were then applied to utilize molasses as substrate for citric acid production. Presence of heavy metals (specially Cu, Zn, Fe and Mn) and phosphate in sufficient amount in the crude molasses made its use difficult, as heavy metals and phosphates were inhibitory for citric acid accumulation. The crude molasses was therefore subjected to pretreatment. The 0.1% hexacyanoferrate treated molasses gave comparable yield with pure sucrose as substrate provided the sugar concentration and pH of the HCF-treated molasses were 12% and 4.5, respectively.

Besides submerged and surface fermentation, solid state surface fermentation (SSF) was also tried for citric acid production from sugarcane molasses with bagasse as solid support. Of the fermentation systems used, SSF was found to be superior, giving nearly 80-85% sugar conversion to citric acid in 9-10 days under optimum fermentation conditions. The multiple cycle SSF system was also tried and found to be advantageous as nearly twice the citric acid production was achieved in the same period and a semi-continuous process can be operated for nearly 20-25 days using the same fungal mat.

Attempts were also made to use the immobilized whole cells of *A. niger* KCU520 for the continuous production of citric acid. Both, calcium- alginate bead and polyacrylamide gel (PAG) entrapment methods were employed for the whole cell immobilization. With calcium-alginate immobilized cells of *A. niger* KCU520, about 65% carbohydrate conversion to citric acid was obtained using a multiple cycle fermentation system in a single-stage bioreactor in 7 days. Half life of the immobilized cells was found to be 40 days. Using a single-stage bioreactor system, nearly 60mg/100ml/h citric acid production was achieved under optimum fermentation conditions with PAG immobilized cells. However, when a two-stage bioreactor was used, approximately 83mg/100ml/h citric acid (20% sugar conversion to citric acid per day) was produced. The PAG immobilized cells remain fully active for nearly 20 days and a semi-continuous fermentation system can be operated for citric acid production. A comparison of various fermentation systems used in this study for citric acid production is summarized in the following table:

TABLE-5. COMPARISON OF VARIOUS FERMENTATION SYSTEMS FOR CITRIC ACID PRODUCTION FROM SUGARCANE MOLASSES USING *A. niger* KCU520.

Fermentation Systems	Citric Acid Yield (% sugar conversion to citric acid)	Volumetric Citric Acid Yield (average sugar conversion to citric acid)
Submerged Fermentation	55-65	5.0
Surface Fermentation	65-75	7.0
Solid state surface Fermentation (SSF)	80-85	9.5
Multiple Cycle SSF	75-80	16.0
PAG Immobilized Cells	15-20	8.5
Alginate Immobilized Cells	60-65	9.0

From these results it is clear that multiple cycle SSF batch process is far more superior to other fermentation processes as citric acid and volumetric citric acid yield is quite high. But due to some technical problems this process was not used for scaling-up studies. The immobilized cell system also offers an attractive choice, as volumetric citric acid yield and longevity of cells was quite high, allowing a continuous fermentation. Although, citric acid yield is somewhat low, this process has the potential for continuous citric acid production in scaled-up bioreactors.

A 10 litre fermenter was designed for scaling-up studies using SSF system and 80-85% sugar conversion to citric acid was achieved in 9-10 days under optimum fermentation conditions. The SSF process for citric acid production seems to be techno-economically feasible.

7.0 REFERENCES

1. Abbot, B.J. (1976) : Preparation of pharmaceutical compounds by immobilized enzymes and cells. In : *Advances in Applied Microbiology* (D. Perlman, ed.) **Vol.20**. Academic Press Inc., New York, pp.203-257.
2. Alter, J.E. and Blumberg, R. (Miles Lab. Inc.) (1981) : Extraction of citric acid. US Patent **4251671** *(Chem. Abstr.* **95**, 61507 d).
3*. Amelung, H. (1930) : Wachstum und saurebildung von *Aspergillus niger* unter wasser. *Chem. Ztg.* **54**, 118 (cf. Doelger and Prescott, 1959).
4. Baniel, A.M., Blumberg, R. and Hajdu, K. (IMI (TAMI) Institute for Research and Development) (1976) : Recovery of acids from aqueous solutions. Br. Patent **1426018** (*Chem. Abstr.* **40**, 132807 e).
5. Banik, A.K. (1975) : Fermentative production of citric acid by *Aspergillus niger.* Strain selection and optimum culture conditions for improved citric acid production. *J. Food Sci. Technol.* **12**, 111-114.
6*. Batti, M.A. (1966) : Process for production of citric acid. US Patent **3290227** (cf. Lockwood, 1975).
7*. Bernhauer, K. (1928) : Zum chemismus der citronensaurebildung durch pilze. I. Die saurebildung aus verschiedenen kohlenstoffver bindungen. II. Die citronensaurebildung aus gluconsaure. *Biochem. Z.* **197**, 309-342 (cf. Prescott and Dunn, 1959).
8*. Bernhauer, K. (1940) : Oxydative garungen In : *Handbuch der Enzymologie* (F.F. Nord and R. Weidenhagen, eds.) Akademische Verlagsgesellschaft, Leipzig (cf.Rohr et al.,1983).
9. Bernhauer, K., Knobloch, H. and Iglauer, A. (1941) : Formation of acid from sugar by *Aspergillus niger.* XI. Factors determining the accumulation of citric acid. *Biochem. Z.* **309**, 151-178.
10. Berry, D.R., Chmiel, A. and Al-Obaidi, Z. (1977) : Citric acid production by *Aspergillus niger* In : *Genetics and Physiology of Aspergillus* (J.E. Smith and J.A. Pateman, eds.) Academic Press, London, pp.405-426.
11*. Brit. Intell. Obj. Sub-committee (BIOS) (1946) : Final report **220**, Production of citric acid at the factory of J.A. Benckiser, Ladenberg, near Heidelberg, 32 Bryanston Square, London, W.I. (cf. Johnson, 1954).

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12. Brodelius, P. (1978) : Industrial applications of immobilized biocatalysts. In : *Advances in Biochemical Engineering* (T.K. Ghose, A. Fiechter and N. Blakebrough, eds.) **vol.10**. Springer, Berlin, Heidelberg, New York, pp.75-129.

13. Bruchmann, E.E. (1961) : Enzymatic studies on mold metabolism. II. Inhibition of aconitase and its significance for citric acid accumulation by *Aspergillus niger* in submerged culture. *Biochem. Z.* **335**, 199-211.

14*. Bruchmann, E.E. (1966) : Action of hydrogen peroxide on the accumulation of citric acid by *Aspergillus niger. Naturwissenschaften* **53**, 226-227 (cf. Kapoor et al., 1982).

15. Cahn, F.J. (1935) : Citric acid fermentation on solid materials. *Ind. Eng. Chem., Ind. Ed.* **27**, 201-204.

16. Chaudhary, K., Chakarvorty, S.C. and Tauro, P. (1972) : Inhibition of fungal growth by sugarcane molasses. *J. Res. Haryana Agric. Univ.* **1**, 48-52.

17. Chaudhary K., Lakshminarayana, K., Ethiraj, S. and Tauro, P. (1978) : Citric acid production by *Aspergillus niger* from Indian cane molasses by solid state fermentation. *J. Ferment. Technol. Jpn.* **56**, 554-557.

18. Chibata, I . (1978) : Immobilized microbial cells with PAA-gel and K-carrageenan and thier industrial applications. *ASC Symp. Ser.* **106**, *Immobilized Microbial Cells* (K. Venkatasubramanian, ed.), pp.187-202.

19. Chibata, I. and Tosa, T. (1977) : Transformation of organic compounds by immobilized microbial cells. In : *Advances in Applied Microbiology* (D. Perlman, ed.) **Vol.22**. Academic Press Inc., New York, pp.1-27.

20. Chibata, I., Tosa, T. and Sato, T. (1974) : Immobilized aspartase containing microbial cells. Preparation and enzymic properties. *Appl. Biochem. Biotechnol.* **27**, 878-885

21. Chmiel, A. (1975a) : Kinetic studies on citric acid production by *Aspergillus niger.* Part I. Phases of mycelium growth and product formation. *Acta Microbiologica Polonica Ser.* **B7(24)**, 185-193.

22. Chmiel, A. (1975b) : Kinetic studies on citric acid production by *Aspergillus niger.* Part II. Two-stage processes. *Acta Microbilogica Polonica Ser.* **B7(27)**, 237-242.

23. Choudhary, A.Q. and Pirt, S.J. (1966) : The influence of metal complexing agents on citric acid production of *Aspergillus niger. J. Gen. Microbiol.* **43**, 71-81.

24. Chung, B.H. and Chang, H.N. (1988) : Aerobic fungal cell immobilization in a dual hollow-fiber bioreactor : continuous production of citric acid. *Biotechnol. Bioeng.* **32**, 205-212.

25*. Cimerman, A., Johanides, V. and Skafar, S. (1976) : Effect of native polymer additions on pellet differentiation in submerged citric acid fermentation. In : *Abstracts of fifth International Fermentation Symposium* (H. Dellweg, ed.), Berlin, pp.405 (cf. Berry et al., 1977).

26. Clark, D.S. (1962) : Submerged citric acid fermentation of ferrocyanide-treated cane molasses. *Biotechnol. Bioeng.* **4**, 17- 21.

27. Clark, D.S. (National Research Council of Canada)(1964) : Citric acid production. US Patent **3118821** (*Chem. Abstr.* **61**, 4926 d).

28. Clark, D.S., Ito, K. and Horitsu, H. (1966) : Effect of manganese and other heavy metals on submerged citric acid fermentation of molasses. *Biotechnol. Bioeng.* **8**, 465-471.

29. Clark, D.S., Ito, K. and Tymchuk, P. (1965) : Effect of potassium ferrocyanide on the chemical composition of molasses mash used in the citric acid fermentation. *Biotechnol. Bioeng.* **7**, 269-278.

30. Clark, D.S. and Lentz, C.P. (1961) : Submerged citric acid fermentation of sugar beet molasses : effect of pressure and recirculation of oxygen. *Can. J. Microbiol.* **7**, 447-453.

31. Currie, J.N. (1917) : The citric acid fermentation of *Aspergillus niger. J. Biol. Chem.* **31**, 15-37.

32. Das, A. (1972) : Strain selection in citric acid fermentation - A review. *Current Science* **41**, 593-596.

33. Das, A. (1980) : Parasexual hybridization and citric acid production by *Aspergillus niger. Eur. J. Appl. Microbiol. Biotechnol.* **9**, 117-119.

34. Das, A. and Nandi, P. (1965) : Production and characterization of ultraviolet induced mutations in *Aspergillus niger. Trans. Bose Res. Inst.* **28**, 83-91.

35. Das, A. and Nandi, P. (1969) : New strains of *Aspergillus niger* producing citric acid. *Experientia* **25**, 1211-1213.

36. Das, A. and Nandi, P. (1969) : Improved production of citric acid from *Aspergillus niger* by mutagenic treatment. *Indian J. Exp. Biol.* **7**, 275-276.

37. Das, A. and Roy, P. (1978) : Improved production of citric acid by a diploid strain of *Aspergillus niger. Can. J. Microbiol.* **24**, 622-625.

38. Das, A. and Roy, P. (1981) : Rapid strain selection for citric acid production. In : *Advances in Biotechnology* (M.Moo-Young, ed.) **Vol 1**, Pergamon Press, Toronto, pp.51-55.

39. de Melle, I.U. (1963) : Process for extracting carboxylic acids produced by fermentation. Br. patent **936339** (*Chem. Abstr.* **57**, 15620 a).

40. Diller, V.M., Tytell, A.G., Tytell, A.A. and Kersten, H. (1950) : A soft X-ray mutant of *Aspergillus niger* van Tieghem. *Plant Phys.* **25**, 340-353.

41. Doelger, W.P. and Prescott, S.C. (1934) : Citric acid fermentation. *Ind. Eng. Chem.* **26**, 1142-1149.

42. Eikmeier, H. and Rehm, H.J. (1984) : Production of citric acid with immobilized *Aspergillus niger*. *Appl. Microbiol. Biotechnol.* **20**, 365-370.

43. Eikmeier, H., Westmeier, F. and Rehm, H.J. (1984) : Morphological development of *Aspergillus niger* immobilized in Ca-alginate and k-carrageenan. *Appl. Microbiol. Biotechnol.* **19**, 53-57.

44. Fencl, Z. and Leopold, J. (1957) : Mechanism of inhibition by acetic acid of the germination of spores of *Aspergillus niger*. *Nature (London)* **179**, 922.

45. Fencl, Z. and Leopold, J. (1959) : Inhibition of spore germination of the mould *Aspergillus niger* by acetic acid. *Folia Microbiol.* **4**, 7-15.

46. Fencl, Z. and Vintrova, E. (1963) : A new metabolic regulator of citric acid production. *Nature (London)* **199**, 905-908.

47. Foster, J.W. (1939) : The heavy metal nutrition of fungi. *Botan. Rev.* **5**, 207-239.

48. Foster, J.W. (1949) : Citric acid. In : *Chemical Activities of Fungi.* Academic Press, New -York, pp.378-418.

49. Foster, J.W. and Davis, H. (1949) : Detection and occurrence of acid-producing fungi. *Bull. Torrey Bot. Cl* **76**, 174-179.

50. Fried, J.H. and Sandza, J.G. (Stauffer Chemical Company) (1959) : Production of citric acid. US Patent **2910409** (*Chem. Abstr.* **54**, 7063 b).

51. Gabriel, B.L. (1982) : *Biological Scanning Electron Microscopy.* Van Nostrand Reinhold Company, New York, Toronto, London, Melbourne.

52*. Gaden, E.L. (1955) : *Chem. Ind. (N.Y.)* **12**, 154 (cf. Rohr et al., 1981).

53. Gardner, J.F., James, L.V. and Rubbo, S.D. (1956) : Production of citric acid by mutants of *Aspergillus niger*. *J. Gen. Microbiol.* **14**, 228-237.

54*. Gold, W. and Kieber, R.J. (1967) : US Patent **3326774** (cf. Rohr et al., 1983).

55. Gomez, A., Schuabel, I. and Garrido, J. (1988) : Pellet growth and citric acid yield of *Aspergillus niger* 110. *Enz. Microb. Technol.* **10**, 188-192.

56. Gupta, J.K., Heding, L.G. and Jargensen, O.B. (1976) : Effect of sugars, pH and ammonium nitrate on formation of citric acid by *Aspergillus niger. Acta Microbiol. Acad. Sci. Hung.* **23**, 63-67.

57. Hackel, U., Klein, J., Megnet, R. and Wagner, F. (1975) : Immobilization of microbial cells in polymeric matrices. *Eur. J. Appl. Microbiol.* **1**, 291-293.

58. Hang, Y.D. and Woodams, E.E. (1984) : Apple Pomace : A potential substrate for citric acid production by *Aspergillus niger. Biotechnol. Lett.* **6**, 763-764.

59. Hang, Y.D. and Woodams, E.E. (1985) : Grape pomace : A novel substrate for microbial production of citric acid. *Biotechnol. Lett.* **7**, 253-255.

60. Henrich, M. and Rehm, H.J. (1982) : Formation of gluconic acid at low pH-values by free and immobilized *Aspergillus niger* cells during citric acid fermentation. Eur. *J. Appl. Microbiol. Biotechnol.* **15**, 88-92.

61. Horitsu, H., Adachi, S., Takahashi, Y., Kawai, K. and Kawano, Y. (1985) : Production of citric acid by *Aspergillus niger* immobilized in polyacrylamide gel. *Appl. Microbiol. Biotechnol.* **22**, 8-12.

62. Horitsu, H. and Clark, D.S. (1966) : Effect of ferrocyanide on growth and citric acid production by *Aspergillus niger. Can. J. Microbiol.* **12**, 901-907.

63. Hossain, M., Brooks, J.D. and Moddox, I.S. (1984) : The effect of sugar source on citric acid production by *Aspergillus niger. Appl. Microbiol. Biotechnol.* **19**, 393-397.

64. Hustede, H. and Rudy, H. (Joh. A. Benckiser Gmb H) (1976a) : Manufacture of citric acid by submerged fermentation. US Patent **3941656** (*Chem. Abstr.* **84**, 178222 w).

65. Hustede, H. and Rudy, H. (Joh. A. Benckiser Gmb H) (1976b) : Manufacture of citric acid by submerged fermenation. US Patent 3940315 (*Chem. Abstr.* 85, 3869 f).

66. *ICUMSA Methods of Sugar Analysis* (1964) : (H.C.S. De Whalley, ed.). Elsevier Publishing Company, Amsterdam, London, New-York.

67*. Ilczuk, A. (1967) : Effect of best molasses medium on the yield of citric acid fermentation. *Gazeta Cukrownicza* **75**, 11-13 (cf. Berry et al., 1977).

68. Islam, M.S., Begum, R. and Choudhury, N. (1984) : Semipilot scale studies on citric acid fermentation by a gamma-ray induced mutant of *Aspergillus niger. Biotechnol. Lett.* **6**, 431-435.

69.Islam, M.S., Begum, R. and Choudhury, N. (1986) : Semipilot scale production of citric acid in cane molasses by gamma-ray induced mutants of *Aspergillus niger*. *Enzyme Microb. Technol.* **8**, 469-472.

70. Jack, J.R. and Zajic, J.E. (1977) : The immobilization of whole cells. In : *Advances in Biochemical Engineering* (T.K. Ghose, A. Fiechter and N. Blakebrough, eds.) **Vol.5**. springer, Berlin, Heidelberg, New York, pp.125-145.

71. James, L.V., Rubbo, S.D. and Gardner, J.F. (1956) : Isolation of high acid-yielding mutants of *Aspergillus niger* by a paper culture selection technique. *J. Gen. Microbiol.* **14**, 223-227.

72. Janota-Bassalik, L., Olczyk, C. and Chodorowska, M. (1970) : Searching, in beet molasses, activators or inhibitors for citric acid fermentation. *Acta Microbiologica Polonica Ser.* **B 2**, 137- 140.

73*.Jefferson, W.R. Jr. (1967) : *Biochemistry* **6**, 3479 (cf. Rohr et al., 1983).

74. Johnson, M.J. (1954) : The citric acid fermentation. In : *Industrial Fermentations* (L.A. Underkofler and R.J. Hickey, eds.). Chemical Publishing, Cherry Valley, New-York, pp.420-445.

75. Johnston, J.R. (1974) : Strain improvement and strain stability in filamentous fungi. In : *The Filamentous Fungi* (J.E. Smith and D.R. Berry, eds.) **Vol.1**, Edward Arnold, London, pp.59-78.

76. Kapoor, K.K., Chaudhary, K. and Tauro, P. (1982) : Citric acid. In : *Prescott and Dunn's Industrial Microbiology* (G. Reed, ed.). 4th Ed., AVI, Westport, C.T., pp.709-747.

77*.Karow, E.O. (1942) : *The production of citric acid in submerged culture.* Doctor's Thesis, Rutgers University, New Brunswick, N.J. (cf. Johnson, 1954).

78. Karow, E.O. and Waksman, S.A. (1947) : Production of citric acid in submerged culture. *Ind. Eng. Chem.* **39**, 821-825.

79. Khan, M.A.A., Hussain, M.M., Khalique, M.A. and Rehman, M.A. (1970) : Methods of citric acid fermentation from molasses by *Aspergillus niger*. *Pak. J. Sci. Ind. Res.* **13**, 439-444.

80. Kiel, H., Guvrin, R. and Henis, Y. (1981) : Citric acid fermentation by *Aspergillus niger* on low sugar concentration and cotton waste. *Appl. Environ. Biol.* **42**, 1-4.

81. Kierstan, M. and Bucke, C. (1977) : The immobilization of microbial cells, subcellular organells, and enzymes in calcium alginate gels. *Biotechnol. Bioeng.* **19**, 387-397.

82. Kirimura, K., Yaguchi, T. and Usami, S. (1986) : Intraspecific protoplast fusion of citric acid producing strains of *Aspergillus niger*. *J. Ferment. Technol.* **64**, 473-479.

83. Kirimura, K., Nakajima, I., Lee, S.P., Kawabe, S. and Usami, S. (1988) : Citric acid production by the diploid strains of *Aspergillus niger* obtained by protoplast fusion. *Appl. Microbiol. Biotechnol.* **27**, 504-506.

84. Kisser, M., Kubicek, C.P. and Rohr, M. (1980) : Influence of manganese on morphology and cell wall composition of *Aspergillus niger* during citric acid fermentation. *Arch. Microbiol.* **128**, 26- 33.

85. Kitos, P.A., Campbell, J.J.R. and Tomlinson, N. (1953) : Influence of temperature on the trace elements requirements for citric acid production by *Aspergillus niger*. *Appl. Microbiol.* **1**, 156-159.

86. Klein, J. and Wagner, F. (1978) : Immobilized whole cells. In : *Proc. First European Congress on Biotechnology* **Vol.82**, Verlag Chemie, Weinheim, New-York, pp.142-164.

87*. Kluyver, A.J. and Perquin, L.H.C. (1933) : *Biochem. Z.* **266**, 68 (cf. Rohr et al., 1983).

88*. Kovats, J. (1946) : The influence of temperature on the rate of citric acid fermentation by *Aspergillus niger*. *Przemyzl Chemiczny* **2**, 122-128 (cf. Berry et al., 1977).

89*. Kovats, J. and Sackowska, L. (1976) : Effect of interruption in aeration of medium in deep citric acid fermentation on molasses solution. *Przem. Ferment. i Rolny* **20**, 22-25 (cf. Berry et al., 1977).

90. Kresling, E.K. (1936) : Morphological and biochemical characteristics of *Aspergillus niger* strains which differ in acid yields. *Proc. Inst. Sci. Res. Food Ind.* **3**, 130-145.

91. Kristiansen, B. and Charley, R. (1981) : Continous process for production of citric acid. In : *Advances in Biotechnology* (M.Moo- Young, ed.) **Vol.I**, Pergamon Press, Toronto, pp.221-227.

92. Kubicek, C.P., Hampel, W. and Rohr, M. (1979) : Manganese deficiency leads to elevated amino acid pools in citric acid accumulating *Aspergillus niger*. *Arch. Microbiol.* **123**, 73-79.

93. Kubicek, C.P. and Rohr, M. (1977) : Influence of manganese on enzyme synthesis and citric acid accumulation in *Aspergillus niger*. *Eur. J. Appl. Microbiol. Biotechnol.* **4**, 167-178.

94. Kubicek, C.P. and Rohr, M. (1978) : The role of the tricarboxylic acid cycle in citric acid accumulation by *Aspergillus niger*. *Eur. J. Appl. Microbiol. Biotechnol.* **5**, 263-271.

95. Kubicek, C.P. and Rohr, M. (1980) : Regulation of citrate synthase from the citric acid accumulating fungus, *Aspergillus niger. Biochim. Biophys. Acta* **615**, 449-457.

96. Kubicek, C.P. and Rohr, M. (1986) : Citric acid fermentation. *CRC Crit. Rev. Biotechnol.* **3**, 331-373.

97. Kubicek, C.P., Zehentgruber, O., El-kalak, H. and Rohr, M. (1980) : Regulation of citric acid by oxygen : Effect of dissolved oxygen tension on adenylate levels and respiration. *Eur. J. Appl. Microbiol. Biotechnol.* **9**, 101-116.

98. Lakshminarayana, K., Chaudhary, K., Ethiraj, S. and Tauro, P. (1975) : A solid state fermentation method for citric acid production using sugar cane bagasse. *Biotechnol. Bioeng.* **17**, 291-293.

99*. Leopold, H. and Valtr, Z. (1961) : Uber einen die citronensauer production durch *Aspergillus niger* stimulierenden faktor in melasse. *Zentralblatt fur Bakteriologie II Abteilung* **114**, 45-46 (cf. Berry et al., 1977).

100. Leopold, H. and Valtr, Z. (1969) : Effect of potassium ferrocyanide on preparation of molasses solution for citric acid fermentation. V. New boiling process. *Nahrung* **13**, 11-19.

101. Linko, P. (1980) : Enzyme engineering in food processing. In : *Food Process Engineering* (P. Linko, Y. Malkki, J. Olkku and J. Larinkari, eds.) **Vol.2**, Appl. Sci. Publ., London, pp.27-39.

102. Linko, P. and Linko, Y.Y. (1984) : Industrial applications of immobilized cells. In : *CRC Critical Reviews in Biotechnology* (G.G. Stewart and J. Russel, eds.) **Vol.I**, pp.289-339.

103. Lockwood, L.B. (1975) : Organic acid production. In : *The Filamentous Fungi* (J.E. Smith and D.R. Berry, eds.) **Vol.I**, *Industrial Microbiology.* Edward Arnold, London, pp.140-157.

104. Lockwood, L.B. and Schweiger, L.B. (1967) : Citric acid and itaconic acid fermentations. In : *Microbial Technology* (H.J. Peppler, ed). Reinhold Publishing, London, pp.183-199.

105. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) : Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.

106. Ma, H., Kubicek, C.P. and Rohr, M. (1985) : Metabolic effects of manganese deficiency in *Aspergillus niger.* Evidence for increased protein degradation. *Arch. Microbiol.* **141**, 266-268.

107.Maddox, I.S., Spencer, K., Greenwood, J.M., Dawson, M.W. and Brooks, J.D. (1985) : Production of citric acid from sugars present in wood-hemicellulose using *Aspergillus niger* and *Saccharomycopsis lypolytica. Biotechnol. Lett.* **7**, 815-819.

108*.Mallea, O. (1950) : La industria de fermentation citrica en La Argentina. *Industriay Quimica* **12**, 264-280 (cf. Lockwood, 1975).

109.Manomani, H.K. and Sreekantiah, K.R. (1987) : Studies on the conversion of cellulose hydrolysate into citric acid by *Aspergillus niger. Process Biochem.* **22**, 92-95.

110.Marrier, J.R. and Boulet, M. (1958) : Direct determination of citric acid in milk with an improved pyridine-acetic anhydride method. *J. Dairy Sci.* **41**, 1683-1692.

111.Martin, S.M. (1955) : Effect of ferrocyanide on growth and citric acid production of *Aspergillus niger. Can. J. Microbiol.* **1**, 644-652.

112.Martin, S.M. (1957) : Citric acid production by submerged fermentation. *Ind. Eng. Chem.* **49**, 1231-1232.

113.Martin, S.M. and Steel, R. (1955) : Effect of phosphate on production of organic acid by *Aspergillus niger. Can. J. Microbiol.* **1**, 470-472.

114.Martin, S.M. and Waters, W.R. (1952) : Production of citric acid by submerged fermentation. *Ind. Eng. Chem.* **44**, 2229-2233.

115*.Miles Lab. (1962) : Citric acid. Br. Patent **908024** (cf. Kapoor et al., 1982).

116.Millis, N.F., Trumpy, B.H. and Palmer, B.M. (1963) : The effect of lipids on citric acid production by an *Aspergillus niger* mutant. *J. Gen. Microbiol.* **30**, 365-379.

117.Milsom, P.E. and Meers, J.L. (1985) : Citric acid. In : *Comprehensive Biotechnology* (M.Y. Murray, ed.) **Vol.3**, Pergamon Press, Oxford, pp.665-680.

118*.Mosbach, K. and Mosbach, R. (1966) : *Acta Chem. Scand.* **20**, 2807 (cf. Jack and Zajic, 1977).

119.Moyer, A.J. (1953a) : Effect of alcohols on the mycological production of citric acid in surface and submerged culture. I. Nature of the alcohol effect. *J. Appl. Microbiol.* **1**, 1-7.

120.Moyer, A.J. (1953b) : Effect of alcohols on the mycological production of citric acid in surface and submerged culture. II. Fermentation of crude carbohydrates. *J. Appl. Microbiol.* **1**, 7-13.

121. Muliskova, M., Panos, J., Sedmera, P. and Fencl, Z. (1980) : Accumulation of 2-Oxo acids in mutants of *Aspergillus niger* reducing lysine. *Folia Microbiol.* **25**, 324-327.

122.Naguchi, Y. (1962) : Effect of trace metals on the production of citric acid by *Aspergillus niger. Hakko Kogaku Zasshi* **40**, 171- 178.

123.Naguchi, Y. and Bando, Y. (1960) : Formation of oxalic acid in the citric acid fermentation in the methanol added molasses medium. *Hakko Kogaku Zasshi* 38, 485-488.

124*.Noyes, R. (1969) : Citric acid production processes. *Noyes Devel. Corp.*, Park Ridge, New Jersey, USA (cf. Rohr et al., 1983).

125.Ohlson, S., Larsson, P.O. and Mosbach, K. (1979) : Steroid transformation by living cells immobilized in calcium alginate. *Eur. J. Appl. Microbiol. Biotechnol.* 7, 103-110.

126.Olbrich, H. (1963) : Molasses. In : *Principles of Sugar Technology* (P. Honig, ed.), Elsevier, Amsterdam, pp.511-696.

127*.Perlman, D. (1947) : Citric acid production by various strains of *Aspergillus niger*. *Rep. Proc. 4th Congr. Int. Microbiol.*, pp. 576 (cf. Gardner et al., 1956).

128.Perlman, D., Dorrell, W.W. and Johnson, M.J. (1946) : Effect of metallic ions on the production of citric acid by *Aspergillus niger*. *Arch. Biochem.* **11**, 131-143.

129.Perlman, D. and Sih, C.J. (1960) : Fungal Synthesis of Citric, Fumaric and Itaconic acids. In : *Progress in Industrial Microbilogy* (D.J.D. Hockenhull, ed.) **Vol.II**, Heywood, London, pp.167-194.

130*.Perquin, L.H.C. (1938) : *Bijdrage tot de kennis der oxydatieve dissimilatie van Aspergillus niger van Tieghem.* Doctor's Thesis, Technical Hochschule, W.D.: Meinema, Delft (cf. Berry et al., 1977).

131. Porges, N. (1932) : Citric acid fermentation by *Aspergillus niger*. *Am. J. Botan.* **19**, 559-567.

132.Prescott, S.C. and Dunn, C.G. (1959) : The citric acid fermentation. In : *Industrial Microbiology*, 3rd Ed., McGraw-Hill Book Co., New-York, pp.572-605.

133.Protod'yakonov, O.P. and Kresling, E.K. (1935) : Relations between acid forming power and morphological characteristics of *Aspergillus niger* strains. *Proc. Inst. Sci. Res. Food Ind.* 3, 101-113.

134*.Quilico, A., Panizzi, L. and Visconti, M. (1949) : Richerche sulla fermentazione citrica con mutanti indotti di *Aspergillus niger*. *R.C. Accad. Lincci* **6**, 40 (cf. Gardner et al., 1956).

135.Rohr, M. and Kubicek, C.P. (1981) : Regulatory aspects of citric acid fermentation by *Aspergillus niger*. *Process Biochem.* **16**, 34- 37.

136.Rohr, M., Kubicek, C.P. and Kominek, J. (1983) : Citric acid. In : *Biotechnology* (H.J. Rehm and G. Reed, eds.) **Vol.3**, Verlag Chemie, Weinheim, pp.420-454.

137.Rohr, M., Stadler, P.J., Salzbrunn, W.O.J. and Kubicek, C.P. (1979) : An improved method for characterization of citrate production by conidia of *Aspergillus niger. Biotechnol. Lett.* **1**, 281-286.

138.Rohr, M., Zehentgruber, O. and Kubicek, C.P. (1981) : Kinetics of biomass formation and citric acid production by *Aspergillus niger* on pilot plant scale. *Biotechnol. Bioeng.* **23**, 2433-2445.

139.Sanchez-Marroquin, A. Carreno, R. and Ledezma, M. (1970) : Effect of trace elements on citric acid fermentation by *Aspergillus niger. Appl. Microbiol.* **20**, 888-892.

140.Shcherbakova, E. Ya. (1963) : Ultraviolet induced variability of *Aspergillus niger. Mikrobiologiya* 32, 43-49.

141. Shu, P. (1953) : Oxygen-uptake in shaken flask fermentations. *J. Agaric. Fd. Chem.* **1**, 1119-1122.

142.Shu, P. and Johnson, M.J. (1947) : Effect of composition of the sporulation medium on citric acid production by *Aspergillus niger* in submerged culture. *J. Bacteriol.* **54**, 161-167.

143.Shu, P. and Johnson, M.J. (1948a) : The interdependence of medium constituents in citric acid production by submerged fermentation. *J. Bacteriol.* **56**, 577-585.

144.Shu, P. and Johnson, M.J. (1948b) : Citric acid production by submerged fermentation with *Aspergillus niger. Ind. Eng. Chem.* **40**, 1202-1205.

145.Snell, R.L. and Schweiger, L.B. (Miles Lab. Inc.) (1951) : Citric acid fermentation. Br. Patent **653808** (*Chem. Abstr.* **45**, 8719 a).

146. Sodeck, G., Modl, J., Kominek, J. and Salzbrunn, G. (1981) : Production of citric acid according to the submerged fermentation. *Process Biochem.* **16**, 9-11.

147.Steel, R., Lentz, C.P. and Martin, S.M. (1955) : Submerged citric acid fermentation of sugar beet molasses : increase in scale. *Can. J. Microbiol.* **1**, 299-311

148.Steel, R., Martin, S.M. and Lentz, C.P. (1954) : A standard inoculum for citric acid production in submerged cultures. *Can. J. Microbiol.* **1**, 150-157.

149.Steinberg, R.A. (1935) : The nutritional requirements of the fungus, *Aspergillus niger. Bull. Torrey Botan. Club.* **62**, 81-90.

150*.Sussman, A.S. and Halvorson, H.O. (1966) : *Spores : Their Dormancy and Germination.* Academic Press, New York (cf. Kapoor et al., 1982).

151.Svenska Sockerfabriks, A.B. (1964) : A method of producing citric acid. Br. Patent **951620** (*Chem. Abstr.* **60**, 2304 a).

152*.Szucs, J. (1944) : Method of producing citric acid by fermentation. US patent **2353771** (cf. Kapoor et al., 1982).

153.Thom, C. and Currie, J.N. (1916) : *Aspergillus niger* Group. Oxalic-acid production of species of *Aspergillus. J. Agr. Res.* **7**, 1-5.

154. Tomlinson, N., Campbell, J.J.R. and Trussell, P.C. (1950) : The influence of zinc, iron, copper and manganese on the production of citric acid by *Aspergillus niger. J. Bacteriol.* **59**, 217-227.

155. Tomlinson, N., Campbell, J.J.R. and Trussell, P.C. (1951) : The influence of zinc, iron, copper and manganese on the production of citric acid by *Aspergillus niger.* II. Evidence for essential nature of copper and manganese. *J. Bacteriol.* **61**, 17-25.

156*.Tressl Von, R., Jakob, R., Kossa, T. and Brown, W.K. (1976) : Gaschromatographisch-massenspektrometrische untersuchung fluchtiger inhall-stoffie von melasse. *Branntweinwirtschaft,* **116**, 117-119 (cf. Milsom and Meers, 1985).

157.Trumpy, B.H. and Millis, N.F. (1963) : Nutritional requirements of an *Aspergillus niger* mutant for citric acid production. *J. Gen. Microbiol.* **30**, 381-393.

158.Tsay, S.S. and To, K.Y. (1987) : Citric acid production using immobilized conidia of *Aspergillus niger* TMB 2022. *Biotechnol. Bioeng.* **29**, 297-304.

159.Vaija, J., Linko, Y.Y. and Linko, P. (1982) : Citric acid production with alginate-bead entrapped *Aspergillus niger* ATCC 9142. *Appl. Biochem. Biotechnol.* **7**, 51-57.

160.Verhoff, F.H. and Spradlin, J.E. (1976) : Mass and energy balance analysis of metabolic pathways applied to citric acid production by *Aspergillus niger. Biotechnol. Bioeng.* **18**, 425-432.

161.Vieth, W.R. and Venkatasubramanian, K. (1976) : Immobilized whole cells in complex biocatalysis. In : *Immobilized Microbial Cells* (K. Venkatasubramanian, ed.), ACS Symp. Ser. **106**, American Chemical Society, Washington, pp.1-11.

162.Vieth, W.R. and Venkatasubramanian, K. (1978) : Immobilized cell system. In : *Enzyme Engineering* (G.B. Broun, G. Manecke and L.B. Wingard, eds.) Vol.**4**, Plenum, New-York, pp.307-316.

163.Von Loesecke, H.W. (1945) : A review of information on mycological citric acid production. *Chem. Eng. News.* **23**, 1952- 1959.

164*.Waller, G.J. (1946) : *Brit. Intell. Obj. Sub-committee (BIOS) Target C22/541 (a), London* (cf. Rohr et al., 1983).

165.Wells, P.A. and Herrick, H.T. (1938) : Citric acid industry. *Ind. Eng. Chem., Ind. Ed.* **30**, 255-262.

166*.Wendel, D.G. (1967) : Diffusion of citric acid in molasses solution, possibilities of technical use for surface culture fermentation. *Zucker* **20**, 238-246 (cf. Kapoor et al., 1982).

167*.Wennerston, R. (1980) : A new method for purification of citric acid by liquid-liquid extraction. In : *Proc. Inst. Solvent Extr. Conf.* **Vol.2**, Assoc. Ind. Univ. Liege, Liege, pp. 80, 63 (cf. Milsom and Meers, 1985).

168.Whitaker, A.J. and Long, P.A. (1973) : Fungal pelleting. *Process Biochem.* **8**, 27-31.

169.Xu, D.B., Kubicek, C.P. and Rohr, M. (1989) : A comparison of factors influencing citric acid production by *Aspergillus niger* grown in submerged culture and on filter paper. *Appl. Microbiol. Biotechnol.* **30**, 444-449.

* **Due to nonavailability of old literature in English and availability of some patented information, various review articles were consulted to seek the information and used as cross references.**