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School of Chemical and Bio Engineering

PRODUCTION OF CITRIC ACID BY ASPERGILLUS NIGER USING MOLASSES AS SUBSTRATE

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ACRONYMS

AAS	Atomic Absorption Spectroscopy
ANOVA	Analysis of Variance
AOAC	Association of Official Agricultural Chemists
BDL	Below detection limit
$CaCl_2$	Calcium chloride
CaO	Calcium oxide
℃	Degree centigrade
CO_2	Carbon dioxide
g	gram
g/L	gram per liter
h	Hours
HCl	Hydrochloric acid
H_2O	Water molecule
H_2SO_4	Sulphuric acid
IS	Indian standard
L	Litres
m^3	Meter cube
mL	Milliliter
$MgSO_4$	Magnesium sulphate
NaOH	Sodium hydroxide
N	Normality
NH_4NO_3	Ammonium nitrate
rpm	Revolution per minute
SSF	Solid State Fermentation
TCA	Tricarboxylic acid
UV	Ultraviolet
v/v	volume per volume

ABSTRACT

Citric acid is an organic acid with numerous industrial application. It is extensively used in the food and beverage industry as it combines a pleasant taste with low toxicity and sweetness. It can be produced from different materials like molasses that has sucrose content of 32%. In Ethiopia, some of the molasses were used for ethanol production and other purposes, but the remaining has been exported to abroad. This study investigated that the utilization of molasses to use as a raw material for citric acid production under different fermentation conditions with submerged fermentation method in the laboratory using 250mL Erlenmeyer flask as the small scale laboratory fermentor by using *Aspergillus niger*. The effect of temperature (25, 30, and 35℃), fermentation time (96, 144, 196h) and pH (2, 4, and 6) of fermentation condition for citric acid yield test result were investigated. The experimental design was done by using Design Expert 6.0.8 software for three factors and Response Surface with 3- level factorial design type in optimization study. The minimum citric acid yield was found to be 20.23 g/L after the fermentation time of 96h, temperature 25℃ and pH 2. A maximum citric acid yield of 40.08 g/L was achieved under selected optimal condition of (fermentation time 156h, temperature 30℃ and pH 3.89) with high value of combined desirability. From the model regression equation developed, the linear terms of temperature, and time had a positive effect and a pH had a negative effect on response yield. The quadratic terms (pure and interaction quadratic terms) had a negative effect on extraction yield except fermentation time and pH interaction. Incubation time had a more weighty linear effect on yield as compared to fermentation temperature and pH. The quality of the product obtained under optimized condition was assessed with Atomic Absorption Spectroscopy (Buck of Scientific 210 VGP AAS, USA) and UV visible Spectrophotometer. The results revealed that the heavy metal content of the sample was below detection limit except Iron and purity of 99.2% respectively. The study indicated that utilization of molasses for citric acid production is considered not only as a positive option in terms of reducing imported citric acid, but also, as an attractive option in means of providing job opportunity and minimizing environmental pollution.

1 INTRODUCTION

1.1 Background

Citric acid ($C_6H_8O_7$, 2-hydroxy-propane-1, 2, 3-tricarboxylic acid) is ubiquitous in nature and exists as an intermediate in the citric acid cycle when carbohydrates are oxidized to carbon dioxide. It is the most important organic acid found virtually in all plants and animals formed by Krebs cycle process (Nadeem *et al*., 2010).It is extensively used in the food and beverage industry as it combines a pleasant taste with low toxicity and sweetness. It serves several functions in the food formulation, like flavor fixation and enhancement, and standardization of acid levels (Knuf, 2014). The use of citric acid in food industry is because of its pleasant acid taste and high solubility in water, preserving and buffering properties in the food and beverages particularly in soft drinks (Kumar and Jain, 2010).It has many applications in pharmaceutical and cosmetic industries as an acidulant, flavour enhancer, preservative, antioxidant, emulsifier and chelating (Nadeem *et al*., 2010; Ramseh and Kalanselvan, 2011).

The annual global production of citric acid has been estimated at about 1.4 to 1.5 million tons per year and its demand is estimated to be growing at a rate of about 3.5 to 4.0% annually (Soccol *et al.*, 2006; Lazar *et al*., 2011).Of the total amount of citric acid produced annually, about 70% is utilized by the food industry; 12% by pharmaceutical industries as flavoring, anticoagulant and preservative while the remaining 18% other industries such as cosmetics, detergent, textile, oil recovery and paper (Soccol *et al*., 2003).Citric acid is also used in the detergent industry as a phosphate substitute, because of less eutrophic effect, and in the cement one to slow down the process of hardening (Max *et al.*, 2010).

Citric acid production synthesis by fermentation is the most economical and widely used way of obtaining this product. More than 90 % of the citric acid produced in the world is obtained by fermentation, which has its own advantages: operations are simple and stable, the plant is generally less complicated and needs less sophisticated control systems, technical skills required are lower, energy consumption is lower and frequent power failures do not critically affect the functioning of the plant. Citric acid production by fermentation can be divided in three phases, which include preparation and inoculation of the raw material, fermentation, and recovery of the product. The industrial citric acid production can be carried in three different ways: These are surface fermentation, submerged fermentation and solid-state fermentation (Papagianni, 2007).The first citric acid fermentation was carried out using *Aspergillus* species in surface fermentation. Surface fermentation refers to the process in which the microorganisms grow as a thick floating mycelial mat over the surface of the liquid media used (Soccol *et al*., 2006).The second methods of citric acid production i.e. submerged fermentation, is the process in which the growth and anaerobic⁄ partially anaerobic decomposition of the carbohydrates by microorganisms in liquid medium occurs with plenty availability of free water (Ray and Ward, 2006). Solid-state fermentation (SSF) is the third microbial technique of citric acid production where cultivation of microorganisms is done in a low-water-activity environmental insoluble materials acting as both nutrient source and physical support (Pandey, 2003; Castilho *et al.*, 2009). In this thesis submerged method of fermentation was selected because it has a better control of the fermentation process and the possible use of a wide range of substrates including glucose, sucrose and cane or beet molasses. However, when compared to others, here was less molasses utilization in Ethiopia at present mainly due to low technological development and low market availability. The use of power alcohol from molasses source for vehicles increases the demand of molasses in most other countries and there is a promising move towards the production and use of power alcohol in Ethiopia also. *Aspergillus niger* was selected for fermentation of molasses in this thesis because the main advantages of using this microorganism are: (a) its ease of handling, (b) its ability to ferment a variety of cheap raw materials, and (c) high yields.

Following the growth of beverage, food, detergent and other factories, its demand has increased from time to time. According to the report of Hailemariam (2010), Ethiopia imports citric acid from different countries like India, Saudi Arabia, China, German, Kenya and USA and expends a lot of money in hard currency. Hence, there is obvious need to achieve industrially sustainable bio-production of citric acid. To meet the rising demand for citric acid in many applications including food and biomedicine, there is a need for continued search for more efficient substrates from environment. Considering this, and the availability of cheap substrates for citric acid production like molasses, a successful citric acid industry must be developed.

1.2 Statement of the Problem

Up to the present time, the Ethiopia's requirement of citric acid is entirely met through import. Its devitrified application in the pharmaceuticals, beverage, food and other industries has been increased the demand annually. As the countries Customs Authority shows the import of citric acid fluctuates from year to year although the general trend is upward. This could be evidenced from the fact that the average imported quantity during the period 2014-2015 is almost double when compared with the period 2005-2006. The yearly average imported quantity during the period 2015-2016 is about 77 tons which is higher compared to the average level of import previously.

So this study could serve as an input to motivate the country to use molasses for citric acid production because of its availability and inexpensive. In Ethiopia approximately about 959810 tons of molasses will be produced annually from eight sugar factors when these factors start to operate fully. But out of this 112574 tons of molasses has been used to produce ethanol. Sugarcane resource can be used to produce a variety of commercial products that can be marketed domestically, regionally and internationally. Ethiopia through its potential in developing large sugarcane production can play a pro-active role in mitigating the same. Molasses the non-crystallizable residue remaining after crystallizing sucrose, has additional advantage; it is relatively inexpensive raw material, readily available and already in use for industrial ethanol production. When compared to others, here was less molasses utilization in Ethiopia at present mainly due to low technological development and low market availability. The use of power alcohol from molasses source for vehicles increases the demand of molasses in most other countries and there is a promising move towards the production and use of power alcohol in Ethiopia also. Keeping in view the future requirements and the availability of cheap raw materials in the country, it would be worthwhile to develop and promote the process of citric acid fermentation, using these locally available materials.

Finally, the rationale of this research work is to investigate the potential use of molasses for citric acid production, which could be a good source of raw material to help meet the future demand of the country.

1.3 Objectives

1.3.1 General objective

The general objective of the study is to utilize molasses as substrates to produce citric acid by *Aspergillus niger*.

1.3.2 Specific objectives

The specific objectives of this study are:

- To characterize some physical and chemical properties of molasses
- To investigate the effect of various factors such as fermentation time, temperature and pH on citric acid yield.
- To determine the optimum operating parameters which maximize the yield.
- To characterize the quality of the product.

1.4 Significance of the Study

- The researches will be able to play an important role by providing a remedy for the environmental pollution due to the discharge of the waste molasses.
- If it is scaled up to large industry, it could create job opportunity and saves foreign currency.
- The import of citric acid could be minimized.

2 LITERATURE REVIEW

2.1 History of citric acid

Before the development of the microbial process, citric acid was commercially produced in England around 1826 from imported Italian lemons (lemons contain 7-9% citric acid) (Max *et al*., 2010).Italian manufacturers had monopoly for its production for almost 100 years, and it was sold at high cost. This led to extensive attempts all over the world to find alternative way for production of citric acid through chemical and microbial techniques.

Until the 1920s, citric acid was extracted from the lemon juice and crystallized (Papagianni, 2007). Extraction from citrus fruits is possible but it is not cheaper than fungal fermentation. However, a small amount of citric acid, approximately less than 1% of the total world production, is still produced from citrus fruits in Mexico and South America where citrus fruits are economically available (Yigitoglu, 1992).

2.2 Microorganisms for citric acid production

A large number of micro-organisms including bacteria, fungi and yeasts have been employed to produce citric acid. Most of them, however, are not able to produce commercially acceptable yields. This fact could be explained by the fact that citric acid is a metabolite of metabolism and its accumulation rises in appreciable amounts only under conditions of drastic imbalances. Kubicek and Rohr (1986) reviewed the strains reported to produce citric acid. Table 2.1 shows the micro-organisms used to produce citric acid. Among these, only *A. niger* and certain yeasts such as *Saccharomycopsis sp.* are employed for commercial production. However, the fungus *A. niger* has remained the organism of choice for commercial production. The main advantages of using this microorganism are: (a) its ease of handling, (b) its ability to ferment a variety of cheap raw materials, and (c) high yields.

Table 2.1 Microorganisms capable of producing citric acid (Manas *et al*., 2011)

Fungi	Yeasts	Bacteria
Aspergillus niger	*Candida tropicalis*	*Arthrobacter paraffinens*
A. aculeatus	*C.oleophila*	*Bacillus licheniformis*
A. carbonarius	*C.guilliermondii*	*Corynebacterium spp.*
C. citroformans	*C. citroformans*	
A. foetidus	*C. intermedia*	
A. luchensis	*C. parapsilosis*	
Penicillium spp.	*C. fibriae*	
Mutant strains	*C. lipolytica*	
A. niger YW-112	*Yarrowia lipolytica*	
A. niger GCB-75	*Hansenula anamola*	

2.3 General characteristics of *Aspergillus* species

Aspergilli are ubiquitous in nature and geographically widely distributed, and have been observed in a broad range of habitats because they can colonize a wide variety of substrates. From 200 genera of molds, *Aspergillus* is the most important filamentous fungus producing white septate hypha which is profusely branched and produces black mass of conidia. The most common species of this genus is *Aspergillus* species and like other fungi, this species also requires an aerobic environment for growth (Asan, 2004). *Aspergillus* species is commonly found as a saprophyte growing on dead leaves, stored grain, compost piles, and other decaying vegetation. The spores are widespread, and are often associated with organic materials and soil.

Fungi generally exhibit four basic growth stages: the spore which is a dormant phase, followed by the spore germination or lag phase, the growth or hyphal phase, and the spore formation phase. During the growth phase, the fungus develops tubular filaments known as hyphae, and these hyphae branch out repeatedly to form a mass known as the mycelium. During this phase, the rate of production of biomass is exponential. Citric acid production is known to occur when fungal growth is limited (Vaija and Linko, 1986). New organisms originate from spores produced asexually by a mature *Aspergillus* species mycelium. To reproduce, fungi develop special hyphae called conidiophore which stand erect and develop numerous spores at their tip. These spores are released into the environment and will remain dormant until proper conditions (moisture, light, temperature, and substrates) allow for their development (Nason, 1968).

The duration of the lag phase depends on several factors, including environmental conditions and level of inoculums (Papagianni, 2004). The lag phase is followed by the exponential growth of the fungus *Aspergillus* species, when growth conditions are favorable and nutrients are available. During exponential growth, hyphae grow in length at their tips, branch out and extend outwards. The exponential growth of the fungal mass may not be uniform within a colony. The older and central hyphae may exhaust their nutrient supply while the hyphae found at the surface may access new sources of nutrients and maintain their growth. When nutrients become limited, especially for the older hyphae, a stationary phase is exhibited where growth is virtually stopped.

2.5 Citric acid production techniques

Industrial citric acid fermentation can be carried in three different ways: by surface, submerged and solid state fermentation, each of these methods requires raw material and inoculum preparation.

2.5.1 Surface fermentation

Liquid surface culture is the classic citric acid production process characterized by low yield, lower energy consumption and less man power (Moyer, 1953). Karklins *etal*. (1996) studied the utilization of different microorganisms in citric acid production by surface fermentation using cane molasses as a substrate. Sakurai *etal*. (1996) studied the effect of oxygen tension on citric acid production by surface culture of *A. niger*. They reported that both yield of citric acid and biomass were almost constant in oxygen tension range of 21 to 74% (v/v). Kilie *etal*. (2001) observed that $MgSO_4$ and KH_2PO_4 in the concentration of 0.25 g/L and NH_4NO_3 in the concentration of 1.5 g/l are optimal for citric acid production by *A. niger* in surface culture. Adham (2002) reported that natural oils with high unsaturated fatty acid content when added at concentrations of 2 and 4 % (v/v) to beet molasses caused a considerable increase in citric acid yield from *A. niger*.

2.5.2 Submerged fermentation

Submerged fermentation has become the method of choice for higher yields of citric acid. Advantages of the submerged method include a better control of the fermentation process and the possible use of a wide range of substrates including glucose, sucrose and cane or beet molasses. Some strains of *A. niger* which produced citric acid in submerged culture, failed to do so when grown on wheat bran in solid state fermentation and vice-versa (Shankaranand and Ramesh, 1992). The optimal conditions reported for citric acid yield were: initial pH, 3.5; temperature, 30 ºC; incubation period, 8 days and sucrose concentration of 150 g/l. Kamzolova *etal*. (2003) found that in the continuous cultivation of *Yarrowialipolytica*, oxygen requirements for growth and citric acid synthesis are dependent on the iron concentration in the medium. Lotfy *etal*. (2007a) found that beet molasses and corn steep liquor were suitable substrates for citric acid production by *A. niger* in submerged culture. El-Hussein *etal*. (2009) used Kenana Sugar Factory cane molasses for citric acid production by *A. niger* using submerged technique. Optimum fermentation parameters reported were: initial pH, 3.5; temperature, 30 ºC; addition of ethanol, 4%; and an initial sucrose concentration, 150 g/l. Papagianni *etal*. (1998) described the relationship between *A. niger* morphology and citric acid production in a tubular loop and a stirred tank bioreactors. The workers also identified a morphology parameter that was used to characterize the process performance in citric acid fermentation. Different cultural conditions such as aeration rate and addition of antifoaming agent were found to influence citric acid production in the culture broth using 15.0 L stainless steel stirred fermentor (Kamil *etal*., 2002). Haq *etal*. (2004) compared different strains of *A. niger* for citric acid production using clarified cane molasses as a basal substrate. Among the cultures, the mutant strain*A.niger* NGGCB-101 gave maximum citric acid production (g/L) compared to that produced by the parental strains. They reported that the addition of $CaCl_2$ to the culture medium promoted the formation of small round fluffy pellets, which were desirable for citric acid productivity.

2.5.3 Solid state fermentation

Solid-state fermentation, also known as Koji process, was first developed in Japan where abundant raw materials such as fruit wastes and rice bran are available. It is the simplest method for citric acid production and is an alternative method for using agro-industrial residues (Vandenberghe *etal*. 2000). Solid-state culture is characterized by the development of microorganisms in a low-water activity on an insoluble material that acts both as physical support and source of nutrients .The yield of citric acid was defendant on the amount of methanol present, the fermentation time and incubation temperature. Maddox *et al*., (1995) studied citric acid production by *A. niger* in solid state fermentation using kumara and taro as substrates, maximum citrate production rates were observed after 2 to 3 days of fermentation. Addition of methanol at 3 – 4 % markedly increased citric acid formation from the waste. Kumar and Jain (2008) reported that treated sugar cane bagasse supplemented with sucrose medium was a better substrate than untreated bagasse carrier. Bari *etal*. (2009) developed citric acid production from oil palm empty fruit bunches by *A. niger* using solid-state technique. As sequential optimization based on statistical design and one factor at time method was employed to optimize the medium constituents for the improvement of citric acid production.

2.6. Economic aspects

Although the surface production process is from the viewpoint of energy requirements, a less expensive, there are a lot of disadvantages in it. This involves larger space requirements for production and isolation, higher steam requirement and higher sterility requests. One of the greatest problems of this production process sterility. Main advantages of the submerged fermentation process are: shorter fermentation time (6 - 7 days), higher level of process sterility and control of process parameters, simpler process operations, lower space requirements, process reproducibility and higher yields.

Schierholt compared the economy of surface and submerged fermentation process for the citric acid production. Capacities of $300m^3$ and $150m^3$ in 9 days of fermentation time at productivity 72 tons and 12 tons per day were compared. On his work he concludes that the building investment costs connected with the surface fermentation process are 2.5 times higher than those connected with the submerged fermentation. Contrary to this, the expenses on equipment are considerably higher at submerged fermentation, and more than 60 per cent of those expenses consist of complicated component as are bioreactors and more sophisticated instrumental control, which are subject to relatively high wear.

The total investment costs for the submerged process are about 25 per cent lower for higher capacities and 15 per cent lower for smaller capacities than for surface fermentation. The more favourable total investment costs for the submerged process are in contrast to considerably higher production costs for any capacity. Especially evident is the high consumption of electric energy, which is about 30 per cent higher as much as that required at surface fermentation. The labour costs in highly developed countries are for surface fermentation considerably higher.

On countries where cooling water temperature exceeds 20℃, additional expenses for cooling the bioreactors are incurred by installation of cooling aggregates for submerged process. The submerged fermentation is sensitive to short interruptions or breakdowns in aeration, which results not only in loss of yield, but also in total breakdown of the respective batch. At surface fermentation, the resulting citric acid solution or fermentation broth is much more concentrated than at submerged fermentation, effected by higher evaporation rates during fermentation.

2.7 Physical properties of citric acid

Citric acid is a colourless white crystalline powder which is practically odourless. It exists as anhydrous $C_6H_8O_7$, molecular weight 192.12 or monohydrate $C_6H_8O_7.H_2O$, molecular weight 210.14. It is solid at room temperatures, melts at 153 °C and decomposes at higher temperature (Kubrick, 1998) boiling point 175 °C. Anhydrous citric acid is highly soluble in water is freely soluble in ethanol and sparingly soluble in ether; monohydrate citric acid is soluble in water and sparingly soluble in ether.

2.8 Citric acid world production

Development of citric acid fermentation industry during the nearly passed century has aroused a great deal of interest. Formerly, the raw material, calcium citrate, was produced almost entirely from citrus products, Italy being by far the largest producer.

Table 2.2 World's main citric acid manufacturers and country head quarter (Markit, 2015).

Company	Country headquarter
Gadot Biochemical Industries	Israel
Weifang Ensign Industry	China
Huangshi Xinghua Biochemical	China
RZBC	China
Anhui COFCO Biochemical	China
Cargill	USA
ADM	USA
Citrique Belge	Belgium
Jungbunzlauer	Switzerland
Tate & Lyle	UK

2.9 Raw materials for the production of citric acid

Several raw materials such as hydrocarbons, starchy materials and molasses, have been employed as substrates for commercial submerged citric acid production. Generally, citric acid is produced by fermentation using inexpensive raw material, including crude natural products, such as hydrolysed starch, sugar cane broth and by-products like sugar cane and beet molasses. Cane molasses is a major byproduct of the sugar industry (Yansong *et. al*. 2000).There are various types of molasses which depends on the source from which they are obtained Rao (1997); beet molasses, cane molasses, black strap molasses, refinery molasses and high test molasses are among the common ones.

Molasses can be converted into many value-added products by application of modern technologies. Many products can be made theoretically Rao (1997), but in actual practice, the production of only a few products is commercially viable and hence, commercial scale plants are working in different countries to produce; ethyl alcohol, baker's yeast, torula yeast protein molasses, L-lysine, acetone-butanol, citric acid, lactic acid, glutamic acid and mono sodium glutamate. The industrial use of molasses arises from its sugar constituents. When compared to others, here was less molasses utilization in Ethiopia at present mainly due to low technological development and low market availability. The use of power alcohol from molasses source for vehicles increases the demand of molasses in most other countries and there is a promising move towards production and use of power alcohol- in Ethiopia also.

Molasses is preferably used as the source of sugar for microbial production of citric acid due to its relatively low cost and high sugar content. Since it is a by-product of sugar refining, the quality of molasses varies considerably, and not all types are suitable for citric acid production. The molasses composition depends on various factors like the variety of beet and cane, methods of cultivation, and conditions of storage and handling such as transportation and temperature variations. Molasses contains about 45-50% total sugars, of which 30-33% are cane sugar and the rest are reducing sugars. Both beet and cane molasses are suitable for citric acid production, however, beet molasses is preferred to sugarcane due to its lower content of trace metals, although there are considerable yield variations within each type. Molasses is a desirable raw material for citric acid fermentation because of its availability and the relatively low price (Xie and West TP. 2006).

2.10 Molasses production in Ethiopia

Previously Ethiopia has three large sugar factories, which can produce molasses as by-product that are methehara, Fincha, and Wonji/Shoa sugar factories. (*Ethiopian Stastisc Authority,* 2006) reported average annual molasses production is 40,000-50,000 tons per year among the sugar factories in Ethiopian. The annual capacity of molasses production in Ethiopia's sugar factories are shown in the table 2.3.

Table 2.3 Tons of molasses production in Ethiopia

No	Factories	Status	Crushing capacity (TCD)	Crushing day per year	Cane to be crushed per year (Ton)	Molasses production per year (Ton)
1	Wonji/Shewa	Operational	6,250	210	1115625	39046.875
2	Metehara	Operational	5,372	242	1105020.4	38675.714
3	Fincha	Operational	12,000	207	2111400	73899
4	Tendaho	Under Commissioning	13,000	250	2762500	96687.5
5	Kesem	Under Commissioning	6,000	242	1234200	43197
6	Arjo Didesa	Under Commissioning	8,000	207	1407600	49266
7	Omo-Kurazi-1	Under Commissioning	12,000	220	2244000	78540
8	Omo-Kurazi-2	Under Commissioning	12,000	220	2244000	78540
9	Omo-Kurazi-3	Under Project	12,000	220	2244000	78540
10	Omo-Kurazi-5	Under Project	12,000	220	2244000	78540
11	Belese-1	Under Project	12,000	207	2111400	73899
12	Belese-2	Under Project	12,000	207	2111400	73899
13	Welkayit	Under Project	24,000	220	4488000	157080
Total						**959,810.089**

Source Ethiopian sugar coorporation, 2017

2.11 Medium composition

Growth of microorganisms and accumulation of citric acid are strongly affected by the medium composition such as type and concentration of the carbon source, nitrogen, phosphorous, potassium, trace elements and stimulators. Certain nutrients need to be in excess (i.e. sugar, protons, oxygen), some have to be limiting (i.e. nitrogen, phosphate) and some others (i.e. trace metals, especially manganese) have to remain below defined limits (Papagianni, 2007).Thus citric acid productivity by *A. niger* can be improved by optimizing medium components.

2.11.1 Carbon source

The type and concentration of carbon source greatly influence the accumulation of citric acid (Vandenberghe, 2003). The presence of easily metabolized carbohydrates has been found essential for good production of citric acid. Sucrose was the most favourable carbon source followed by glucose, fructose and galactose.

2.11.2 Nitrogen source

Citric acid production is directly influenced by the concentration and nature of the nitrogen source. Nitrogen starvation during fermentation limits the growth of *A. niger* and enhances citric acid production (Haq *etal*. 2005).The concentration of nitrogen source required for citric acid fermentation is 0.1 to 0.4 N /liter. Ammonium salts, urea, peptone and malt extract were the most suitable nitrogen sources for production of citric acid by the fungus

2.11.3 Phosphorous source

The concentration of exogenous phosphorous in the fermentation medium has a significant effect on cell multiplication and metabolites production. Low levels of phosphate have a positive effect on citric acid production. The presence of excess phosphate leads to a decrease in the fixation of carbon dioxide, which in turn increases the formation of certain sugar acids and the stimulation of growth. Rehman et al. (2003) studied different phosphorous sources for citric acid production by *A. niger* using cane molasses. They found that $K_2HPO_4$was the best phosphate source for the process.

2.11.4 Micro-elements

Many enzymes require the presence of non-protein factors, these include complex organic molecules and in many cases simple metal ions A number of divalent metals such as zinc, manganese, iron, copper and magnesium have been found to affect citric acid production by *A. niger*. There is elevated production of citric acid only if a rigorous control of the trace elements availability is accomplished, mainly in the submerged process. On the other hand, the presence of manganese ions and iron and zinc (in high concentrations) could cause the reduction of citric acid yields only in phosphate free medium. As a consequence of this, the addition of chelating agents such as potassium ferrocyanide to the medium proved to be of no use.

2.11.5 Stimulating agents

The supplementation of the fermentation medium with low alcohols or lipid materials, such as vegetable oils and fatty acids, to increase citric acid production has been reported. Appropriate alcohols are methanol, ethanol, isopropanol or methyl acetate. The optimal amount of methanol/ethanol depends upon the strain and the composition of the medium, generally optimum range being 1-3%. Anwar et al. (2009) used different metal complexing agents such as EDTA and potassium ferrocyanide to reduce heavy metal ions during the fermentation process.

2.11.6 Aeration

Citric acid production is an aerobic process; therefore oxygen supply has a determinant effect on its production. Increased rates of oxygen supply lead to enhanced yields and reduced process time. Aeration in the batch fermentation should not be interrupted because it has been found to be quite harmful to the process. Aeration supplies the necessary oxygen to the microorganisms, and agitation maintains uniform conditions within the fermenter. Altogether, the aeration and agitation are important in promoting effective mass transfer to liquid medium in the fermenter. The main function of a properly designed bioreactor is to provide a controlled environment in order to achieve the optimal growth or product formation in the particular cell system employed. In laboratory shake flasks, aeration and agitation are accomplished by the rotary or reciprocating action of the shaker apparatus. In pilot-scale and production-scale fermenters, oxygen is generally supplied by compressed air, and mechanical devices are used to agitate the liquid broth. In aerobic fermentation processes, oxygen is a key substrate, and because of its low solubility in aqueous solutions, a number of studies to enhance the efficiency of oxygen mass transfer have been conducted. The concentration of dissolved oxygen in a suspension of respiring microorganisms generally depends on the rate of oxygen transfer from the gas phase to the liquid, on the rate at which oxygen is transported to the site of utilization, and on the rate of its consumption by the microorganism. In the conventional water soluble carbohydrate substrate processes, it has frequently been found that the rate of oxygen transfer from dispersed air bubbles can become the rate limiting factor by the rate of supply of oxygen.

2.12 Process parameters

2.12.1 Temperature

The significance of temperature in the development of a biological process lies in the fact that it could determine some important effects, such as protein denaturation, enzyme inhibition, acceleration or suppression on the production of a particular metabolite. Nampoothiri *et al*. (2004) Reported that citric acid production could be affected by a slow germination of the fungi, slow metabolic activity, and enzyme denaturation and reduced cell viability when *A.niger* cells are incubated under low or high temperatures.

The optimum temperature for filamentous fungi such as *A. niger* are mesophilic thus requiring optimal temperatures between 25°C and 35°C for growth (Reid, 1998).When the temperature of medium is low, the enzyme activity is also low, giving no impact on the citric acid production at 24ºC (Sikander *et al.,* 2002).But when the temperature of medium is increased above 30ºC, the biosynthesis of citric acid is decreased. Different workers have also used 30ºC as the cultivation temperatures and obtained higher values of actual product (Sikander *et al.,* 2002).

2.12.2 pH

Most filamentous fungi are observed to grow well under slightly acidic conditions, ranging from 3 - 6, but some fungi are able to grow at a pH below 2. The pH is important in two respects during the citric acid production. Firstly, spore germination which is required for fermentation requires pH of 5 and above to occur. Secondly, protons are released when ammonia is absorbed by germinating spores. This causes a release of hydrogen ions thus lowering the pH of the medium. Low pH values *A. niger* citric acid fermentation are desirable for in improving citric acid production, suppresses formation of oxalic acid and providing a close to sterile environment which reduces the risk of contamination (Max *et al.,* 2010). A low pH also inhibits the production of unwanted organic acids (gluconic acid, oxalic acid), and this makes the recovery of citric acid from the broth (Levente and Christian 2003).

2.12.3 Time

The optimal time of incubation for maximum citric acid production varies among microorganisms and type of substrate used as well as fermentation conditions. Many studies revealed that the optimum time course ranges from 3 days to 14 days for citric acid production (Ashour, A. *et al*, 2014).

2.13 Pre-fermentation treatments of substrate

Several attempts have been made to produce citric acid using molasses, which is preferred due its low cost and high sugar content (40-55%). Both, cane and beet molasses are suitable for citric acid production. However, beet molasses is preferred due to its lower content of trace metals. Generally, cane molasses contains calcium, magnesium, manganese, iron and zinc, which have a retarding effect on the synthesis of citric acid. Consequently, some pre-treatment is required for the removal/reduction of trace metals. Because the concentration of trace metals has such a profound effect on citric acid production, various techniques have been used to reduce trace metals in fermentation media (Kristiansen, et al. 1999). Complete elimination is practically impossible, particularly when raw materials such as molasses are used, but two approaches have had some success: 1) chemical pretreatments to reduce trace metal concentrations, and 2) development of fungal strains able to produce high levels of citric acid in the presence of excess trace metals. Potassium ferrocyanide treatment precipitates iron and zinc and has been extensively used. The chemical is either added directly to the fermentation medium, where too much could be inhibitory to fungal growth, or to the substrate (molasses) prior to inoculation.

2.14 Recovery of citric acid

At the end of fermentation, the medium contains citric acid and various undesirable by-products such as mycelium, other organic acids, mineral salts, proteins, etc. The following steps are necessary for the recovery of citric acid from the fermentation medium. Depending on the process used, the first step is either the separation of liquid broth from the mycelium, or the precipitation of oxalic acid. Separation of the fermentation broth from fungal mycelia and cells can be done by filtration or centrifugation, or a combination of the two processes. Mycelium may be washed to recover additional citric acid that can constitute up to 15% of the total production (Kristiansen, et al. 1999). Waste mycelia may also be pressed to recover additional broth (Max, et al. 2010). The next step is the purification of citric acid, which can be accomplished by a number of methods. The six most common methods are: precipitation; solvent extraction; adsorption, absorption and ion exchange; liquid membranes; electro dialysis; and ultrafiltration (Kristiansen, et al. 1999). Precipitation is the most common purification practice. The principle behind the purification methods involves the precipitation of insoluble tricalcium citrate from the fermentation broth. A number of physical factors determine the efficiency of the precipitation process. These include the citric acid concentration, temperature, pH, and rate of lime addition. Milk of lime containing calcium hydroxide/calcium oxide (180-250 g/L) is gradually added while the temperature is maintained at 20°C and the pH is below but close to 7. Loss of citric acid is minimally 4-5% due to solubility of calcium citrate. Most other impurities remain in solution and may be removed by washing the calcium citrate with minimal amounts of water until no sugars, chlorides or colored materials wash off. The calcium citrate is then filtered off and recovered. This is then treated with sulfuric acid (60-70%) to form citric acid at 90℃ and insoluble calcium sulfate (gypsum). The gypsum is filtered off leaving a solution of 25-30% citric acid. This solution may be filtered with activated carbon to remove impurities and/or purified with ion exchange columns. Finally, the liquor is concentrated in vacuum crystallizers at 20-25°C, forming citric acid monohydrate. Crystalization at temperatures higher to this is used to prepare anhydrous citric acid.

The main advantages of precipitation are that it is highly selective, has no phase transition, and has high product purity. Meanwhile, finding proper precipitants for the products is the key factor for this method. As for the industrial-scale calcium precipitation process, when a one molar amount of organic acid is converted, an equal amount of $Ca(OH)_2/CaCO_3$ and H_2SO_4 are consumed, and low valuable calcium sulfate is formed (Lee SC, *et. al.* 2011).

2.15 Uses of citric acid

Low toxicity and a high solubility characteristic of citric acid, makes it suitable for use in food, pharmaceutical and detergent industries.

2.15.1 Uses of citric acid in the food and beverage industry

It is widely used as an acidulant in the food, sugar, and confectionery and beverages. Citric acid is the most versatile and widely used food acidulant. The use of citric acid as a good acidulant depends in part on its strength as an acid and its pleasant taste, while its property of enhancing existing flavour shave ensured its dominant position in the market. The ability of citric acid to complex heavy metals such as iron and copper has led to its increasing use as a stabilizer of oils and fats where it greatly reduces oxidation catalysed by these metals. It is also employed as an aid to emulsification in the manufacture of processed foodstuffs, for example, cheese. In addition, the triethyl, tributyl and acetyltributyl esters of citric acid are employed as non-toxic plasticizers in plastic films used to protect foodstuffs.

2.15.2 Uses of citric acid in medically related aspects

The use of citric acid in the pharmaceutical industry is mainly based on its sequestering action, for example in the stabilization of ascorbic acid and on the effervescent effect it produces when combined with carbonates and bicarbonates, for example in antacid and soluble aspirin preparations. Citric acid is often used as the anion in pharmaceutical preparations which make use of basic substances as the active agent. Trisodium citrate is widely used as a blood preservative, where it prevents clotting by complexing calcium. Ferric ammonium citrate is still used in the treatment of anaemia although other iron salts are increasingly preferred. Mixtures of citric acid and its salts have good buffering capacity and are extensively used for this purpose in the pharmaceutical, toiletry and food industries.

2.15.3 Uses of citric acid in other industries and the environment

Its ability to complex metals combined with its low degree of attack on special steels allows the use of solutions of citric in the cleaning of power station boilers and similar installations. Citric acid has also found uses in electroplating and in the bioremediation of soils contaminated by heavy metals (Ates, *et.al* 2002). In the areas where there are restrictions on the inclusion of phosphates in detergent formulations trisodium citrate is replacing phosphates in specialty cleaners and heavy-duty liquids. In a process for the removal of sulfur dioxide from the flue gases of power stations and metal smelters proposed by the bureau of mines in India, a buffer solution containing principally H_2 Citrate is used as a scrubbing agent.

2.16 Previous work on molasses composition

Composition of molasses varies from country to country and from factory to factory within the same country and from season to season with in a factory depending on the type of cane, soil character, cane age, fertilizer used, chemicals used in processing sugar production.

Table 2.4 Composition of Wanji Shao molasses (Wondimagegen D., 2008)

No	Characteristics	Ethiopian standards(w/v)	John (w/v)	Curtin (w/v)	Laboratory Results (w/v)	Remarks
1	Total solids	85%	79%	79.5%	80%	✓
2	Total reducing Sugar	50%	45-55%	46%	47.7%	✓
3	Reducing Sugar	14%	10-15%	-----	11.16%	✓
4	Nitrogen	---	0.15-.8%	-----	0.88%	✓
5	Calcium	---	0.08-.5%	0.8%	0.42%	✓
6	Ash	14%	8.1%	8.1%	8.3%	✓

- ✓ = Acceptable or within the standard

As it has been observed from Table 2.3 the result of composition analyzed such as total solids, total reducing sugar, reducing sugar, nitrogen and calcium contents of the molasses was in acceptable range as compared to the standard specified.

2.17 Application of citric acid in Ethiopia

Citric acid is used in different industry in Ethiopia the same to other countries because of its pleasant acid taste and its high solubility in water.

Table 2.5 Applications of citric acid

Industry	Applications
Beverages	Provides tartness and complements fruits and berries flavors. Increases the effectiveness of antimicrobial preservatives. Used in pH adjustment to provide uniform acidity.
Jellies, Jams and Preserves	Provides tartness. pH adjustment.
Candy	Provides tartness. Minimizes sucrose inversion. Produces dark color in hard candies. Acts as acidulant.
Frozen fruit	Lowers pH to inactivate oxidative enzymes. Protects ascorbic acid by inactivating trace metals
Dairy products	As emulsifier in ice creams and processed cheese; acidifying agent in many cheese products and as an antioxidant.
Pharmaceuticals	As effervescent in powders and tablets in combination with bicarbonates. Provides rapid dissolution of active ingredients. Acidulant in mild astringent formulation. Anticoagulant.

3 MATERIALS AND METHODS

The experimental work was carried out in Addis Ababa University, Addis Ababa Institute of Technology, Food science and Nutrition department laboratory and Arba Minch University.

3.1 Materials and Chemicals

The major raw material used in the experiment was molasses which has sucrose content of 30-33%. It was acquired from Wanji Shoa Sugar Factory which lies downstream of the Koka Dam in the Central Rift Valley of Ethiopia in the Awash River Basin, 110 km southeast of Addis Ababa and 10 km south of Adama. The sugarcane molasses were collected in clean, durable plastic container and stored at room temperature for further uses.

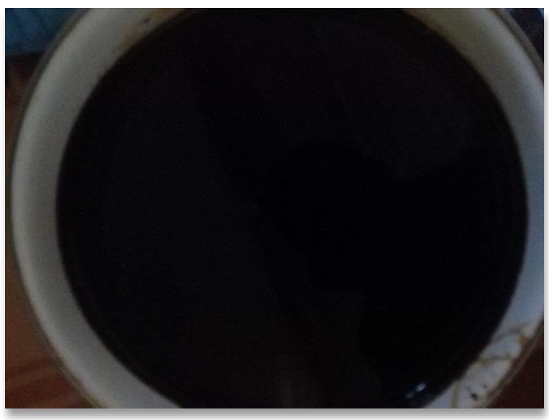


Figure 3.1 Molasses before fermentation

The chemicals and analytical reagents grade used were 0.1M sodium hydroxide, 0.1N sulfuric acid, 1M calcium hydroxide, phenolphthalin as indicator, distilled water, ethanol absolute, activated carbon, potato dexter's agar (OXOID, England) and 1M hydrochloric acid.

Test cultures for fermentation of the molasses was selected from fungal strains, which are common food spoilage and pathogenic microorganisms. The fungal strains used in this investigation was *A.niger* (American type culture collection ((ATCC-25922). This tests organism was obtained from microbiology (traditional medicine) department of Ethiopian Public Health Institute (EPHI).

3.2 Equipment

The equipment used during the experimentations includes laboratory autoclave, magnetic stirrer, Erlenmeyer flasks 250mL, volumetric flask, 100mL, rotary incubator (SHKA4450-1CE), Whatmann (No.42), membrane filter paper (45µm), test tubes 50mL, burette 100mL, balance (precision, ±0.0001 g), oven (MB45, OHAUS, Switzerland), different size conical and beakers 50mL, 100mL,250mL, measuring cylinders 50mL, 100mL,500mL, centrifuge (UNIVERSAL 320 R) , pH meter, water bath (HWS-26), freeze dryer (alpha 1-4 LD, Osterode am Harz, Germany), aluminium foil, gloves, petridis, thermometer, vacuum filter (KIF, LABOPORT), atomic absorption spectroscopy (Buck of Scientific 210 VGP AAS, USA) and Spectrophotometer (T60U model, PG Instruments Ltd).

3.3 Methods

The overall structure of the experimental works was shown in Figure 3.2.

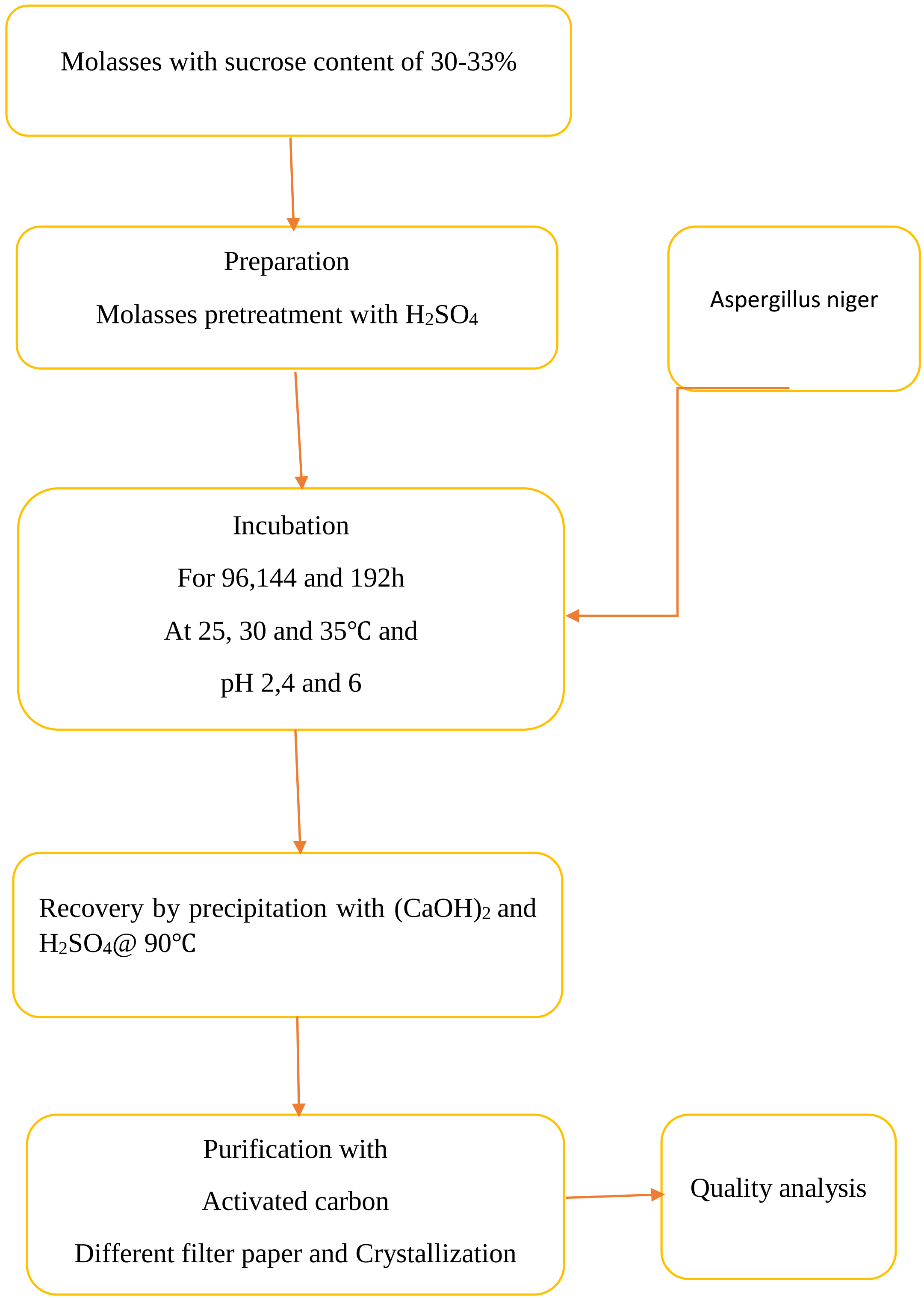


Figure 3.2 Frame work of the experiment

3.4 Biochemical compositions analysis and Physical characteristics of sugarcane molasses

3.4.1 Determination of total solids

Ten gram cane molasses was weighed and well mixed. It was diluted with 50mL of distilled water to bring to 60 g of total weight and stirred with a glass rod until completely dissolved. Then it was filtered through a fluted what man No 41, filter paper covered on the funnel with a watch glass to minimize the evaporation. 20mL of the filtrate collected was discarded and the remaining was used for the determination of refract metric brix at 20℃. The refractive index of the of the molasses solution at 20 ℃ (degree Brix at 20℃) was directly read from the instrument (Ethiopian standard, 2004).

3.4.2 Determination of nitrogen content

Using Kjeldahl method all nitrogen was converted to ammonia by digestion with a mixture of 0.1N concentrated sulphuric acid and concentrated Orthophosphoric acid containing potassium sulphate as boiling point raising agent and selenium as catalyst. The ammonia released after alkalinization with sodium hydroxide was steam distilled into boric acid and titrated with Sulphuric acid. 0.5g molasses sample transferred into a tecator tube, place it in the tecator rack. Then 5 mL of NH_4cl solution was added in to each tecator tubes and 6ml of the acid mixture that was mixed of 5 parts of concentrated Orthophosphoric acid with 100 parts of concentrated sulphuric acid. It has mixed the molasses sample and acid carefully. 3.5 ml of hydrogen peroxide was added step by step. There was a violent reaction by adding 3g of the catalyst mixture for 30minute before digestion. Digestion was going on for more than 3 hour at 370℃. Distillation has been placed on a 250 mL conical flask contain 25 mL of the boric acid indicator solution under the condenser of the distiller with its tip immersed into the solution. Transferred the digested and diluted solution into the sample compartment and added 25 mL of the 40 % sodium hydroxide solution into the compartment, it has been rinsed down with a small amount of water, and switch on the steam. It has been distilled until 100 mL then continued until a total volume of a few ml of water before the receiver was removed. Then it was titrated with 0.1N sulphuric acid to a reddish color using the radio meter pH stat.

Total percent of nitrogen in a sample $= \frac{(T - B) * N * 14 * 100}{W}$ (3.1)

Where; T: Volume in ml of the standard sulphuric acid solution used in the titration for the test

B: Volume in ml of the standard sulphuric acid solution used in the titration for the blank determination.

N: Normality of standard sulphuric acid

W: Weigh in grams of the test material.

3.4.3 Determination of moisture content

The moisture content measurement of molasses was performed by taken 10 grams of molasses sample and oven dried in a crucible at 104℃ for 30 minutes (Hubert, 2006). Then the results were calculated using the following equations:

$$Mc\ (\%, w/w) = \frac{W_2 - W_3}{W_2 - W_1} * 100\% \quad (3.2)$$

Where: M_c – the moisture content (%)

W_1 – weight of crucible (g)

W_2 – weight of sample and crucible (g)

W_3 – weight of dry sample and crucible (g)

Figure 3.3 Oven dryer

3.4.5 Ash content

A dry, tarred porcelain dish containing 10g sample was placed in a muffle furnace set at 550℃ for 2 h, and the sample was cooled in a desiccator and weighed. The ash content was calculated using the equation as described in AOAC (2000):

$$\text{Ash (\%w/w)} = \frac{M_2 - M_1}{M_3} * 100\% \qquad (3.3)$$

Where- M_1 - mass of empty dish, g

M_2 - mass of dish with ash, g

M_3 - mass of fresh sample, g

3.5 Raw material Preparation

3.5.1 Acidic pre-treatment of sugarcane molasses

One major disadvantage in using molasses for citric acid production is its high ash content that contain some trace metals, which inhibits efficient citric acid production. To reduce or eliminate the inhibitory action of metal, appropriate pretreatment is given to the molasses solution. Cane molasses obtained from Wanji Shoa Sugar Factory was pre-treated with 0.1N H_2SO_4 as suggested by Panda et al. (1984). Fifteen milliliters of the cane molasses was diluted up to 100 mL with distilled water. Then 5.0 mL of the acid was added and placed in a water bath at 90℃ for 1 h. After cooling at room temperature, the medium was neutralized with lime water and left to stand overnight for clarification, and the clear liquid supernatant was diluted to 15% sugar level to use as a fermentation medium.

3.13 Design of experiments

In this research citric acid was produced using submerged process method. Data analysis was performed by DESIGN EXPERT software using response surface methodology with 3- Level Factorial method. The response variable is the yield. There were three factors with three levels for each i.e. incubation time (96h, 144h & 192h), temperature (25℃, 30℃&35℃), & pH (2, 4 &6).This design of the experiment would be helpful to differentiate the significance of the main and the interaction factors. The data collected in all cases of experiments was the amount of citric acid expressed in g/L. The results were statistically analyzed using one way analysis of variance

(ANOVA) to find out the optimal conditions for citric acid production. All the experiments were carried out at random in order to minimize the effect of unexplained variability in the observed responses due to systematic errors. The optimization of yield results was carried out by three chosen independent process variables.

A total of 32 experiments were carried out and the data was statistically analyzed by the Design-Expert program to find the suitable model for the citric acid yield as a function of the three variables. Ranges and levels of variables investigated in this research were given in Table 3.2.

Table 3.1 Design Experiments factors and levels

Factors	Levels		
	-1	0	1
Incubation time (h)	96	144	192
Temperature (℃)	25	30	35
pH	2	4	6

3.6 Experimental setup

3.6.1 Fermentation conditions preparation and settings

The experiment was conducted as shown in the Table 3.1. It was conducted under nine (9) different blocks and some of them were repeated based on the response surface methodology 3- Level factors of the experiment.

Table 3.2 Repetitions for the fermentation experiment

Run	Incubation time (h)	Temperature (℃)	Repetitions
1	96	35	3
2	144	35	3
3	192	35	3
4	96	25	3
5	96	30	3
6	144	30	8
7	144	25	3
8	192	25	3
9	192	30	3
Total			**32**

3.6.1.1 Fermentation temperature

Temperature plays very critical role in microbial growth and metabolism. The influence of temperature on citric acid production was studied by carrying the fermentation at different temperatures ranging from 25℃, 30℃ and 35℃ for different incubation time and pH. The three levels of temperature were selected because of the fact that most filamentous fungi are mesophilic and require for growth optimal temperatures between 25 and 35℃ (Suresh *et al.*, 1999)

3.6.1.2 Fermentation time

The optimum time of incubation for maximum citric acid production varies both with the organism and fermentation condition (Kubicek, 1998). The other factor studied in Citric acid production in this study was the incubation period at different pH and temperature. To observe the effect of fermentation it was carried out for various time periods *i.e.* 96, 144, and 192 hours at various temperature and pH per the experimental design.

3.6.1.3 Fermentation pH

To determine the effect of pH on citric acid yield fermentation was carried out at different initial pH (2, 4 and 6) for various temperature and incubation period. After molasses was treated and clarified with sulphuric acid (0.1N H_2SO_4), the pH of the sample was adjusted by sodium hydroxide (0.1M NaOH) and hydrochloric acid (1M HCl) to desired value. These pH levels were selected because previous studies had shown that the production of citric acid by *Aspergillus* species grown successfully with initial pH values ranging from 2 to 6 (Adham, 2002

3.7 Inoculum preparation

A. niger was obtained from microbiology (traditional medicine) department of Ethiopian Public Health Institute (EPHI). It was maintained on potato dextrose agar (pH 5.6) slants. The potato dextrose agar medium was prepared by dissolving 3.9 g of PDA in approximately 80 mL of distilled water and raising the final volume up to 100 mL. This was cooked for 10-15 min while constant stirring until a clear solution formed. The pH was maintained at 5.6 by 0.1N HCl or 0.1 NaOH. Approximately 5.0 ml of this medium was poured into the individual test tubes. The tubes were cotton plugged and sterilized in an autoclave at (121°C) for 15 min. After sterilization, the test tubes were kept in a slanting position (at an angle of about 30°) to increase the surface area. The PDA slants were then inoculated by transferring a small amount of *A. niger* conidia from the culture provided and incubated at 28°C (5 days) for maximum sporulation. After 5days the cultures were stored at 4°C in a lab cool for further studies.

3.8 Fermentation experiment

In this experiment submerged fermentation method was used for the production of citric acid in the laboratory using 250mL Erlenmeyer flask as the small scale laboratory fermentor and all flasks were autoclaved at 121℃ for 20 minutes. Two hundred milliliters of clarified cane molasses was taken in each of the 250mL Erlenmeyer flask; then all the media were autoclave at 115℃ for 10 minutes. After cooling the media at room temperature; an amount of 50 mL of distilled water was added to the fungal pure culture to make a fungal suspension and then 1 mL from this suspension was transferred to the sugarcane molasses media. Then ethanol (3%) was added to the media before fermentation to enhance citric acid production (Nadeem *et al*., 2010). Finally flasks were incubated with KH_2PO_4 in the concentration of 0.25 g/L and NH_4NO_3 in the concentration of 1.5 g/l according to Kilie etal. (2001) at a rotary incubator shaker (SHKA4450-1CE) at 25℃, 30℃ and

35°C at different time and pH and the shaking speed was kept at and 200rpm. After incubation, period was ended the fermentative sample was taken for citric acid extraction and observe the results (Dubey, 2003). The three levels of temperature were select because of the fact that most filamentous fungi are mesophilic and require for growth optimal temperatures between 25 and 35°C (Suresh *et al*., 1999).

Figure 3.4. Rotary incubator

3.9 Separation mechanism

There were different separation mechanism used to recover citric acid from fermented broth. However, in this thesis the precipitation recovery method was used for extraction of citric acid from fermentation media after 4, 6 and 8 days of incubation period. The first stage involved the addition of 1M calcium hydroxide (180 to 250 g/L) gradually at a temperature of 50℃ and pH below, but close to, 7 to accomplish the process of neutralization, which resulted in the formation of a precipitate of calcium citrate (Heding LG and Gupta J. 1975):

$$2C_6H_8O_7 + 3Ca(OH)_2 \longrightarrow (C_6H_5O_7)_2Ca_3.4H_2O + 2H_2O \quad (3.4)$$

After addition of 1M calcium hydroxide, the extract or filtrate was stirred by magnetic stirrer for 20min to allow the calcium citrate to form as a fine, white precipitate as a result of an exothermic reaction. The precipitate was recovered by centrifuging the suspension at 3000 rev/min for 10 min and the precipitated calcium citrate was removed by filtration and washed several times with water.

(a) (b) c) (d)

Figure 3.5 (a) Magnetic stirrer, (b) test tube with fermented media, (c) centrifuge machine and (d) dried calcium citrate.

On the second stage insoluble calcium citrate was converted to soluble citric acid and insoluble calcium sulphate after concentrated 0.1 N sulphuric acid (60%) was added to the calcium citrate based on the stoichiometric relationship in Equation 3.7. Following the addition of sulphuric acid, the mixture of insoluble calcium citrate and sulphuric acid were stirred with magnetic stirrer for at least one hour at 90℃ to allow the calcium sulphate to precipitate.

$$(C_6H_5O_7)_2Ca_34H_2O+3H_2SO_4 \longrightarrow 2C_6H_8O_7 +3CaSO_42H_2O+2H_2O \qquad (3.5)$$

3.6 Calcium sulphate (Gypsum)

Finally the resultant suspension obtained from step two was centrifuged at 3000 rev/min for 10 min and filtered the calcium sulphate with Whatmann No.42 filter paper. Then the filtrate were subjected to Activated Carbon and vacuum filtration for the removal of colorants. Finally the supernatant (presumed to be citric acid in solution) have been filtered by micro membrane filter paper to remove the residues of fungi.

3.10 Citric acid yield determination by titration with NaOH

The concentration of citric acid in supernatant was estimated titrimetrically (AOAC, 1995) by using 0.1 M NaOH and phenolphthalin as indicator. Hence, a known volume of the analyte (citric acid) was placed in a titration flask and the burette filled by a standard solution (0.1M NaOH) until it reached the zero mark using a funnel. Before the titration was start, one to three drops of phenolphthalein was place in the titration flask with the citric acid. A 0.1M NaOH was slowly added from the burette, drop by drop with continual swirling to keep it thoroughly mix. The titration continues, drop by drop, until the phenolphthalein suddenly achieves the intermediate color (weak pink) between that of the acid and the color of the base.

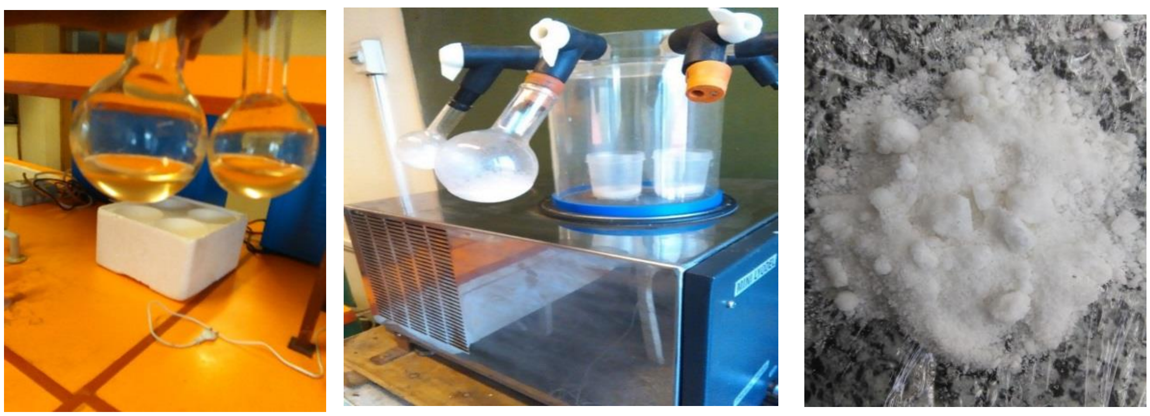
The titration ceases when the system was neither acidic nor basic referred to as the endpoint. The endpoint was corresponding to a perfect stoichiometric relationship between the acid and the base. Once the endpoint has been reached, the volume of NaOH used (titre) on the burette had read and the titration is complete and the final calculations can be done using citric acid, 1mL 0.1M NaOH is equivalent to 0.064 g citric acid (Emeka *et al*.2012) the amount of citric acid can be expressed in terms of g/L citric acid factor (0.064) formula.

$$\frac{g}{L}\text{Acid} = \frac{\text{Titre(volume of NaOH)}*0.064*100*10}{10\ \text{(ml analyte)}*1000\text{ml}} = \text{Titre} * 0.64 \qquad (3.6)$$

Figure 3.7 Citric acid after titrated by NaOH (0.1M)

3.11 Crystallization of citric acid

Freeze drying is often regarded as the best method of water removal to obtain final products of the highest quality. Because of the absence of liquid water and the low temperatures required for the process, most of the deterioration reactions and microbiological activities are prevented, which gives a final product of excellent quality. The mother liquor containing citric acid was finally, concentrated in freeze dryer, and forming citric acid.



(a) (b) (c)

Figure 3.8 (a), (b) and (c) Samples ready for crystallization (b) Freeze dryer and (c) citric acid

3.12 Characterization of the product

3.12.1 Assessment of purity of the product

Citric acid was estimated gravimetrically following the recommended pyridine-acetic anhydride method (Marrier and Boulet, 1958). Three grams of the sample was accurately weighted and dissolved in 40 mL of distilled water, then for each 1mL of sample, 1.3 ml of pyridine and 5.7ml of acetic anhydride were added. The test tube was then placed in a water bath at 32ºC for 30 minutes. The optical density of the sample was measured at 420 nm using UV visible spectrophotometer. The citric acid concentration of the sample was estimated from a reference run in parallel after citric acid standard was prepared from citric acid monohydrate by dehydrating it at 90ºC for 72hours to constant weight.

3.12.2 Determination of Sulphated ash

Silica crucible was dried over a burner for 10 min, cooled in a desiccator containing silica up to room temperature. Then five gram of the sample was weighted accurately in a tared crucible. It was ignited in muffle furnace at 650℃ for 2 hrs as shown in Figure 3.9, gently at first, until the material was thoroughly charred. The residue were cooled, moistened with 1 ml of sulphuric acid and ignited gently till the carbon was completely oxidized. The crucible was cooled in a desiccator and weighted (Ethiopian standard, 2004).

$$\text{Sulphated ash, percent by mass} = \frac{M1}{M2} * 100\% \qquad (3.7)$$

Where: M_1 = mass, in g, of the residue; and

M_2 = mass, in g, of the material taken for the test.

Figure 3.9 Muffle furnace

3.12.3 Preparation of samples for determination of heavy metals

3.12.4.1 Digestion of samples

The sample was prepared for each metal according to Wet-ashing technique developed by Maria, 2002. One gram of the samples was weighed out into acid washed glass round bottom flask. Samples were digested by the addition of 20ml of aquaregia (mixture of HCl and HNO_3, ratio 3:1). Condenser was fitted to the round bottom flask that reflex for two hours at 90℃. The round bottom flask wall was washed with distilled water and the sample was filtered out through whatman filter paper No 1 to separate the insoluble solid from the supernatant liquid. The volume was adjusted to 100ml with distilled water. All samples and blanks were stored in plastic containers. Standard solution of each sample As, Pb, and Fe was prepared according to Sc 2000 manufacturer procedure for Atomic absorption spectroscopy (Buck scientific model 210 VGP AAS, USA and ZEEnit 700, German).

(a) (b)

Figure 3.10 (a) Samples to be digested and (b) Digested samples

3.12.4.2 Determination of metal concentration of each sample

After digested, the sample was connected to AAS to read concentration of the metal ions present in the sample. Then As, Fe & Pb were analyzed with AAS (Buck scientific model 210VGP and ZEEnit 700, German) equipped with deuterium arc back ground corrector and standard air acetylene flame system using external calibration curve after the parameters (burner and lamp alignment, slit width and wave length adjustment) were optimized for minimum signal intensity of the instrument. Three replicate determinations were carried out on each sample. Hollow cathode lamps operated at the manufacturers recommended conditions were used at their respective primary source line. Concentration of the metal ions present in the sample was determined by reading their absorbance using AAS and comparing it on the respective standard calibration curve.

Figure 3.11 (a) Atomic Absorption Spectroscopy

4 RESULTS AND DISCUSSION

4.1 Biochemical composition of cane molasses

In this section, the results of the experiments carried out on molasses such as chemical composition, physical characteristics and the effect of fermentation parameters such as temperature, pH and fermentation time on the amount of citric acid yield through submerged fermentation method, and the products items relationship with imported citric acid was investigated and discussed here under.

The obtained result shows that cane molasses composition presented in Table 4.1, reflect, there was find in the same range result from analysis presented in the following publications (John, 1954 and Ethiopian standards, 2004).

Table 4.1 Summary of laboratory results and comparing with other publication

No	Characteristics	Ethiopian standards (w/v), 2004	John (w/v) John, 1954	Present study (w/v)
1	Total solids	85%	79%	80%
3	Nitrogen	---	0.15-.8%	0.40%
4	moisture content	---	12-20%	14%
5	Ash	14%	8.1%	8.3%

As it has been observed from table 4.1 the total solids, nitrogen content, moisture content and ash content of the molasses were in the acceptable range deduce that it was suitable to use it as substrate to produce citric acid.

4.2 Citric acid yield

The yield of citric acid was calculated by Equation 3.8. According to the experimental design 32 runs were performed and their corresponding extraction yields were demonstrated in the Table 4.2. The extraction yields obtained were ranged from 20.23g/L to 40.98 g/L. The minimum extraction yield was obtained at low levels of extraction parameters. On other hand, the maximum extraction yield was obtained when operating extraction at center level of the factors. The maximum yield obtained was compared with past investigations. The present study was almost in agreement with Sikander *et al*. (2002) who reported that the produced citric acid from molasses was (18.86±1.8) to (42.56±2.0) g/L.

Table 4.2 Yield of citric acid

Run no.	Incubation time(h)	Temperature(℃)	pH	Yield(g/L)
1	96(-1)	25(-1)	2.00(-1)	20.23
2	144(0)	25(-1)	2.00(-1)	30.29
3	192(1)	25(-1)	2.00(-1)	30.21
4	96(-1)	30(0)	2.00(-1)	26.73
5	144(0)	30(0)	2.00(-1)	37.12
6	192(1)	30(0)	2.00(-1)	35.24
7	96(-1)	35(1)	2.00(-1)	24.72
8	144(0)	35(1)	2.00(-1)	35.45
9	192(1)	35(1)	2.00(-1)	33.57
10	96(-1)	25(-1)	4.00(0)	23.92
11	144(0)	25(-1)	4.00(0)	34.84
12	192(1)	25(-1)	4.00(0)	34.29
13	96(-1)	30(0)	4.00(0)	27.98
14	144(0)	30(0)	4.00(0)	40.79
15	192(1)	30(0)	4.00(0)	38.86

16	96(-1)	35(1)	4.00(0)	25.75
17	144(0)	35(1)	4.00(0)	36.92
18	192(1)	35(1)	4.00(0)	35.26
19	96(-1)	25(-1)	6.00(1)	20.54
20	144(0)	25(-1)	6.00(1)	32.36
21	192(1)	25(-1)	6.00(1)	33.84
22	96(-1)	30(0)	6.00(1)	25.33
23	144(0)	30(0)	6.00(1)	36.73
24	192(1)	30(0)	6.00(1)	36.28
25	96(-1)	35(1)	6.00(1)	20.35
26	144(0)	35(1)	6.00(1)	32.74
27	192(1)	35(1)	6.00(1)	32.84
28	144(0)	30(0)	4.00(0)	40.26
29	144(0)	30(0)	4.00(0)	40.85
30	144(0)	30(0)	4.00(0)	40.88
31	144(0)	30(0)	4.00(0)	40.98
32	144(0)	30(0)	4.00(0)	40.45

4.3 Effect of operating conditions on the yield

The effects of individual operating conditions such as temperature, fermentation time and pH on the yield were investigated.

4.3.1 Effects of temperatures on citric acid yield

As shown in Figure 4.1, extraction of citric acid were significantly affected by temperature at fixed center levels of both pH and fermentation time. As illustrated in the Figure 4.1, the extraction yield was increasing as the temperature increased from low to center level from 35.1807 g/L to 40.98g/L as the temperature increases from 25℃ to 30℃, and was slightly decreasing above the center level from 40.98g/L to 36.92g/L to high level of temperature. This is because when the temperature of medium is low, the enzyme activity is also low, giving no impact on the citric acid production and higher temperatures result in enzyme denaturation and inhibition, excess moisture losses and growth arrest (Adinarayana *et al*., 2003). This result was in agreement with the finding of (Rehman *et al*. 2002) who states that an increase or a decrease in the incubation temperature beyond 30℃ has been found to decrease citric acid yield due to denaturation of the enzyme citrate synthase and activation of oxalic acid synthesis pathway. Other researchers also reported 30°C as best temperature for citric acid production (Ali *et al*., 2002; Kareem *et al*., 2010 and Kim *et al*., 2002). It was also reported that 30°C temperature is optimum temperature for citric acid production under submerged condition using Aspergillus species (Nwoba *et al*., 2012).

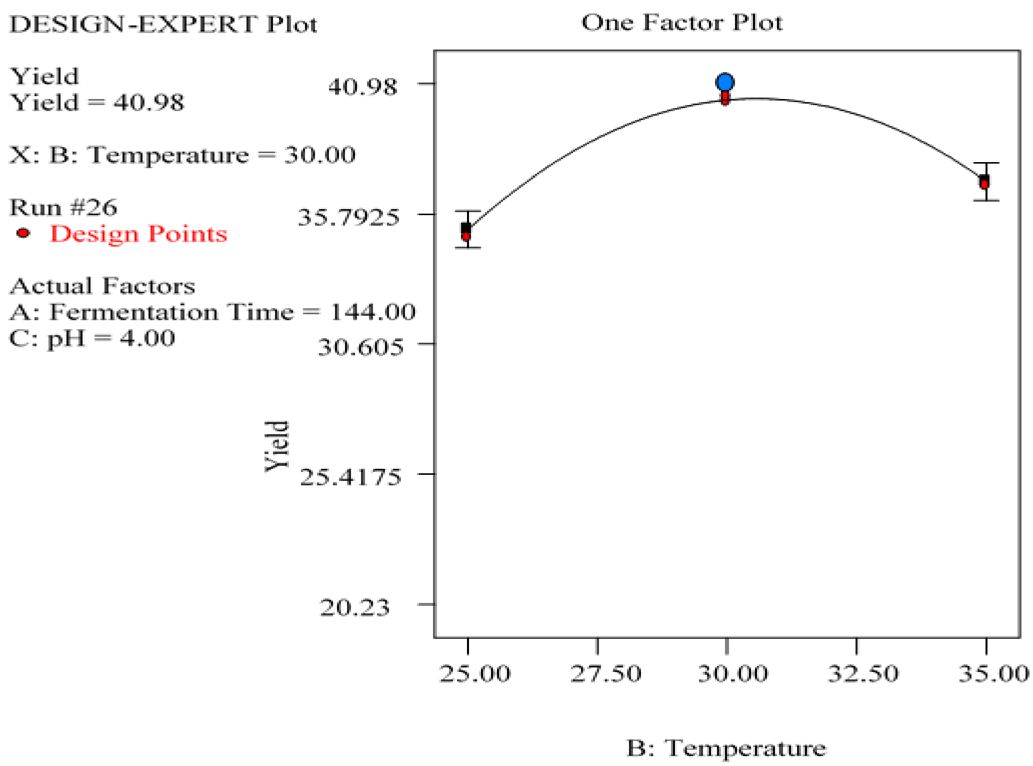


Figure 4.1 Effect of temperature on yield at fixed pH and fermentation time

4.3.2 Effects of fermentation period on citric acid yield

The extraction yield was increased significantly with the increasing in the extraction time at fixed levels of both temperature and pH as illustrated in the Figure 4.2. This study showed that citric acid accumulation increased gradually after 4 days of fermentation from 27.98g/L to 40.98g/L as shown in Figure 4.2. The maximum amount of citric acid (40.98g/L) was obtained at 6 days and there after it showed a slight decrease (38.86g/L) at 8 days of incubation time. This result was comparable with the results of Rehman *et al*. (2003) who reported six days to be optimum for citric acid production using molasses as substrates .Further increase in incubation period did not enhance citric acid production. It might be due to decrease in amount of available nitrogen in fermentation medium, the age of fungi, the presence of inhibitors produced by fungi itself and the depletion of sugar contents. The incubation time required for maximal citric acid production depends on the organism and fermentation conditions (Ishaq *et al.*, 2002).

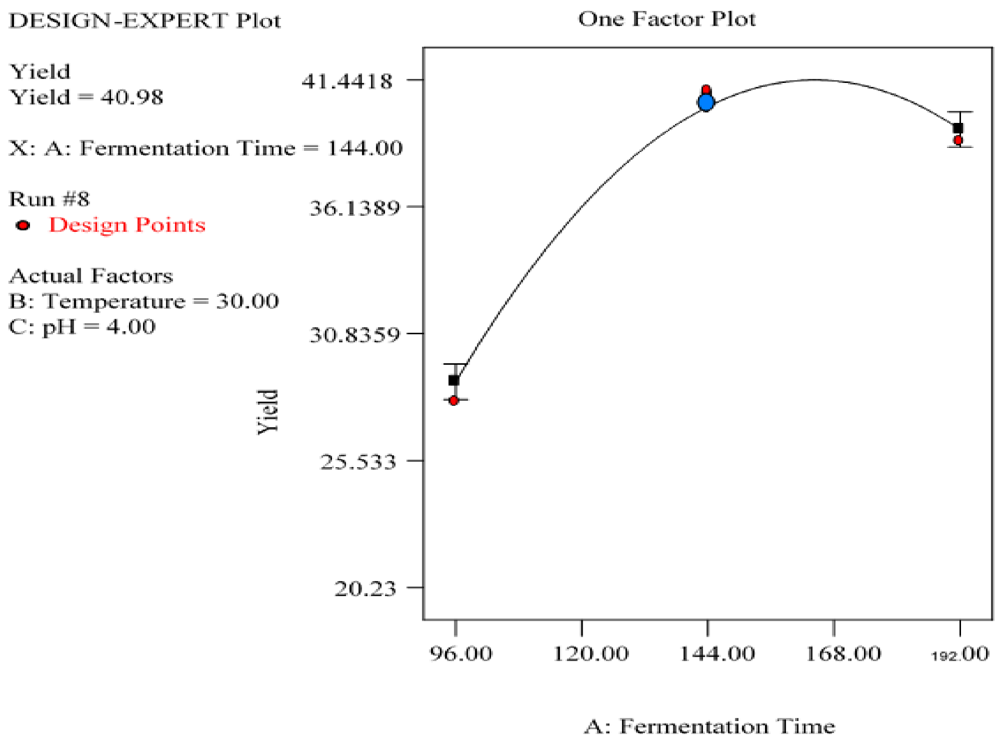


Figure 4.2 Effect of fermentation time on yield at fixed temperature and pH

4.3.3 Effects of pH on citric acid yield

The effect of pH on citric acid production was shown in Fig. 4.3. From the plot as pH increases from 2 to 4 the citric acid yield was increased. Above as well as below this value (pH=4) citric acid amounts were found to decline in the fermentation broth. This is because a low pH in cane molasses medium has been found inhibitory for the growth of *A. niger*. This finding was comparable with the finding of El-Hussein etal. (2009) who reported that the initial pH of 3.5 was optimum for maximum citric acid production using molasses as a fermentation substrate.

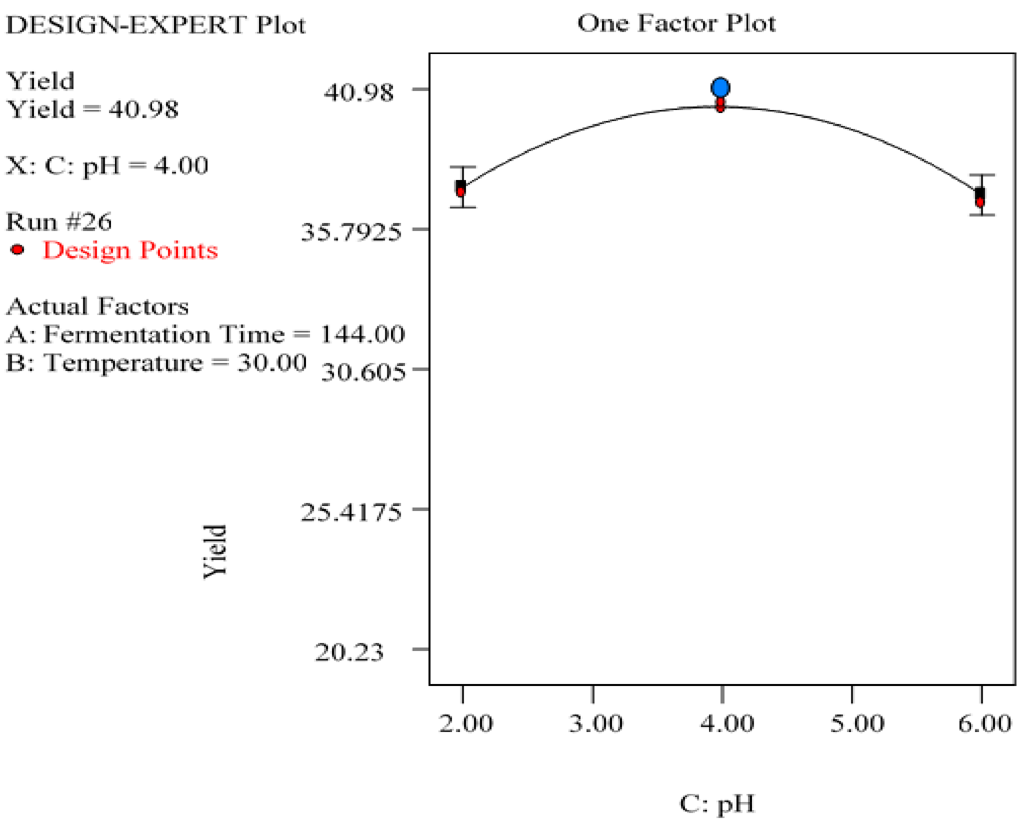


Figure 4.3 Effect of pH on yield at fixed temperature and fermentation time.

4.4 Experimental design analysis

The experimental design selected for analysis of variance was surface methodology (RSM). Under response surface methodology, 3- level factorial was selected. The response of the analysis was extraction yield. The response yield was ranged from 20.23 to 40.98g/L. The ratio of maximum to minimum yield was 2.0257. A ratio greater than 10 usually indicates a transformation is required. For a ratio less than 3, the power transformations have little effect. The aim of model fit summary was maximizing the "adjusted-R square" and the "predicted-R square". Model significance was checked for both model and model factors, linear model factors fermentation time (A), temperature (B) and pH (C) and, quadratic model factors; pure quadratic terms (A^2, B^2, C^2) and interaction quadratic terms (AB, AC, BC) depending on the F and P values.

Table 4.3 Analysis of variance (ANOVA) for the response citric acid yield of molasses

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F
Model	1281.40	9	142.38	475.90	< 0.0001
A-Incubation Period	499.70	1	499.70	1670.28	< 0.0001
B-Temperature	16.21	1	16.21	54.17	< 0.0001
C-pH	0.36	1	0.36	1.21	0.2837
AB	0.65	1	69.77	2.18	0.1536
AC	7.36	1	0.65	24.61	< 0.0001
BC	15.92	1	7.36	53.20	< 0.0001
A^2	277.48	1	277.48	927.51	< 0.0001
B^2	126.07	1	126.07	421.41	< 0.0001
C^2	69.77	1	69.77	233.22	< 0.0001
Residual	6.60	22	0.30		
Lack of Fit	6.20	17	0.36	4.58	0.0503
Pure Error	0.40	5	0.079		
Cor Total	1287.98	31			

As illustrated in Table 4.3, the Model F-value of 475.90 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, A^2, B^2, C^2, AC, BC are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The "Lack of Fit F-value" of 4.58 implies there is a 5.03% chance that a "Lack of Fit F- value" this large could occur due to noise. Non-significant lack of fit is good so the model can be fitted. The model fit summary statistics were listed in Table 4.4.

Table 4.4 Model adequacy measures for citric acid yield

Std. Dev.	0.55	R-Squared	0.9949
Mean	32.71	Adj R-Squared	0.9928
C.V. %	1.67	Pred R-Squared	0.9880
PRESS	15.42	Adeq Precision	66.152

The "Pred R-Squared" of 0.9880 is in reasonable agreement with the "Adj R-Squared" of 0.9928. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. In this case, the ratio of 66.152 indicates an adequate signal. This model can be used to navigate the design space.

4.5 Development of model equation

The application of RSM offers an empirical relationship between the response function and the independent variables. The mathematical relationships between the response and the independent variables fermentation time (A), temperature (B) and pH (C) in terms of coded and actual factors can be determined by Design Expert software. The model equation that correlates the response (Y) to the extraction process variables were given in equation below.

Final equation in terms of coded factors:

$$Yield = + 40.33 + 5.27*A + 0.95*B - 0.14*C - 6.23*A^2 - 4.20*B^2 - 3.12*C^2 - 0.23*A*B + 0.78*A*C - 1.15*B*C \quad (3.11)$$

Where, A = Incubation period

B = Temperature

C = pH

From the regression model equation developed in terms of coded factors, the response yield was affected by linear terms incubation period (A), temperature (B) and pH (C) and, quadratic terms, pure quadratics terms (A^2, B^2, C^2) and interaction quadratic terms (AB, AC, BC). On the basis of the coefficients in equations, it was evident that the response yield increases with the incubation period (A), and temperature (B), which have positive linear effect on extraction yield but incubation period has a more weighty linear effect on yield as compared to temperature. Pure quadratic terms (A^2, B^2, and C^2) has negative effect on the response yield but the effect of pure quadratic term (A^2) has substantial effect than the other quadratic terms. Interaction of Incubation period and pH (AC) has positive quadratic effect on response yield. Interaction of Incubation period and Temperature (AB) and interaction of temperature and pH (BC) have negative quadratic effect on yield.

Final model equation in terms of actual factors:

$$\begin{aligned} Yield = & - 213.88161 + 0.88480*\text{Fermentation Time} + 10.86567*\text{Temperature} + 8.45519*pH \\ & - 2.70313E\text{-}003*\text{Fermentation Time}^2 - 0.16792*\text{Temperature}^2 - 0.78075*pH^2 \\ & - 9.72222E\text{-}004*\text{Fermentation Time}*\text{Temperature} + 8.15972E\text{-}003* \\ & \text{Fermentation Time}*pH - 0.11517*\text{Temperature}*pH \end{aligned} \quad (3.12)$$

Model equations of both coded and actual factors can be used to make predictions about the response for given levels of each factor. The coded model equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

4.6 Model adequacy check

The quality of the model developed could be evaluated from their coefficients of correlation. The regression model was found to be significant with correlation coefficients of R-Squared, adjusted R-Squared and predicted R-Squared having a value of 0.9949, 0.9928, and 0.9880 respectively. Figure 4.4 shows the relation between the actual value of the experiment and the value predicted by the model equation developed by the DESIGN EXPERT 6.0.8 software. The value of R-squared for the developed correlation is 0.9949. It implies that 99.49% of the total variation in the citric acid yield is attributed to the experimental variables studied. The results on Figure 4.4 demonstrated that the regression model equation provided a very accurate description of the experimental data, in which all the points are very close to the line of perfect fit. This result indicates that it was successful in capturing the correlation between the three process variables to the gram per litter yield. Figure 4.4 is the guarantee for the validation of the model equation. The figure shows how the data generated from developed model equation is close to the actual data obtained. The effectiveness of the model could also be measured so as to assure its approximation to the true value. Thus regression coefficient, R^2, could be used for checking its adequacy. The regression value is between [0, 1], and as it approaches to 1. It fits well to the experimental data otherwise it indicates failure of approximation. In this case, R^2 0.9949 was obtained, which was close to 1 and the value Adj- R^2 was 0.9928, and it is in a reasonable agreement with R^2.

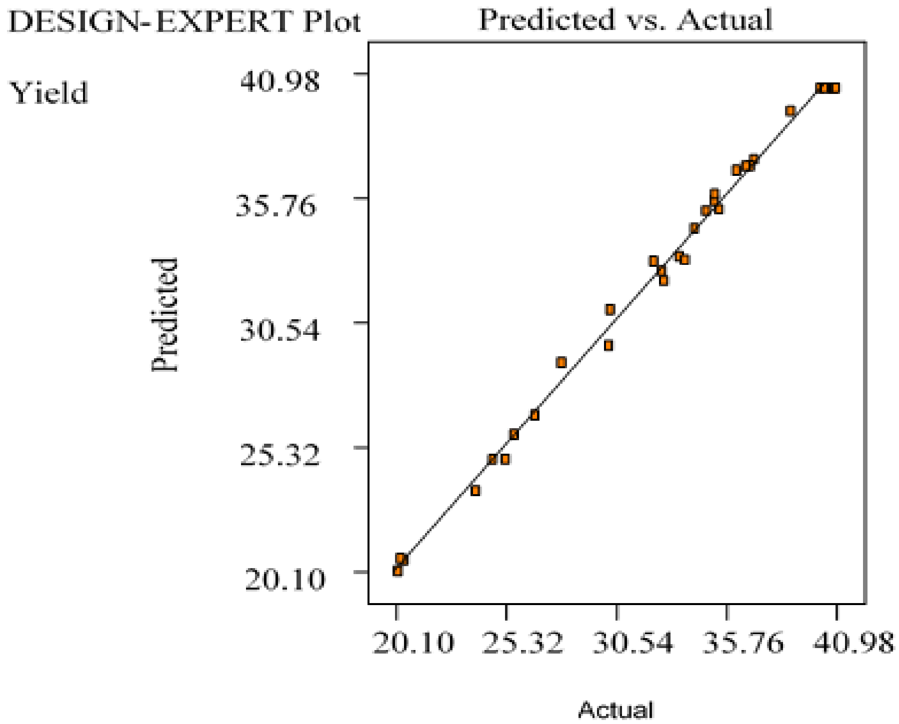


Figure 4.4 Predicted versus actual experimental value for citric acid yield

4.7 Effect of interactive parameters between process variables

The extraction response was also significantly affected by two interactive variables at fixed third variable. The interaction between of the two factors was observed using three-dimensional response surface curves (plotted in order to understand the interactions between the variables and the optimum levels of each variable for maximum yield) and contour curve (presented the effect of two variables) on the yield holding the third variable at constant level. The interaction between two variables namely, temperature and pH at fixed time, temperature and time at fixed pH, pH and time at fixed temperature. The significance of interaction between the corresponding variables was indicated by saddle nature of the contour plots.

An interaction occurs when the response was different depending on the settings of two factors. Plots make it easy to interpret two factor interactions. They would appear with two non-parallel lines, indicating that the effect of one factor depends on the level of the other. If the plotted points fall outside the range, the differences are unlikely to be caused by error alone and can be attributed to the factor effects. If the "I beams" overlap there is no significant difference (95% confidence is default) between the two points. We can then choose the most economical or convenient level for that factor. As it can be seen from Equation 3.11 the interaction factor effects on citric acid yield can be understood easily by the coefficients of interaction factors. There were three interaction factors analyzed by the model equation. These were:

- AB – Fermentation time and temperature
- AC - Fermentation time and pH
- BC – Temperature and pH

Among these three interaction factors interaction of temperature and pH (BC) is the most significant interaction factor for citric acid yield as a response, because it has the highest coefficient (1.15) of the rest. The sign of the coefficient of the interaction factor indicates the effect of interaction factor on citric acid yield. Therefore, the interaction factors with positive signs have positive effect on citric acid yield (as interaction factor increases citric acid yield increases). Whereas, interaction factors with negative signs have a negative effect on citric acid yield (citric acid yield decreases as interaction factor increases). The 3D response surface and contour plots of the effect of interaction of incubation period, temperature and pH with the response of citric acid yield were discussed below.

4.7.1 Effects of fermentation period and temperature on citric acid yield

The interactive effects of temperature and fermentation period on extraction yield were shown in the form of 3D plots and surface contour plots in Figure 4.5 (a) and (b). As it can be observed from figure with rising fermentation period and temperature at fixed pH the citric acid yield was increased but it declined when the fermentation period and temperature increased further. The maximum yield was found at the moderate fermentation period and temperature. This is because when the temperature of medium is low, the enzyme activity is also low, giving no impact on the citric acid production and higher temperatures result in enzyme denaturation and inhibition, excess moisture losses and growth arrest. And also further increase in incubation period did not enhance citric acid production. It might be due to decrease in amount of available nitrogen in fermentation medium, the age of fungi, the presence of inhibitors produced by fungi itself and the depletion of sugar contents.

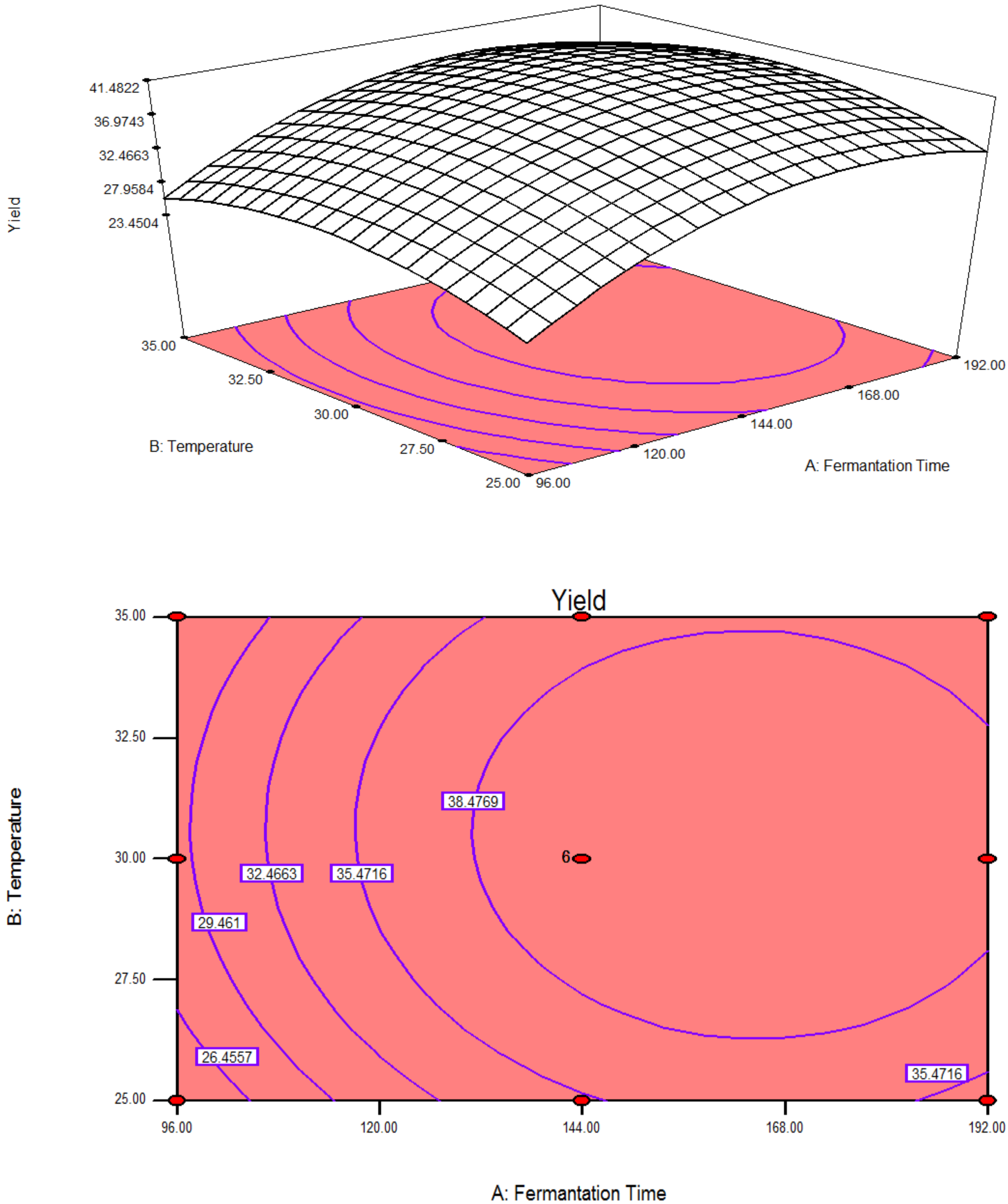
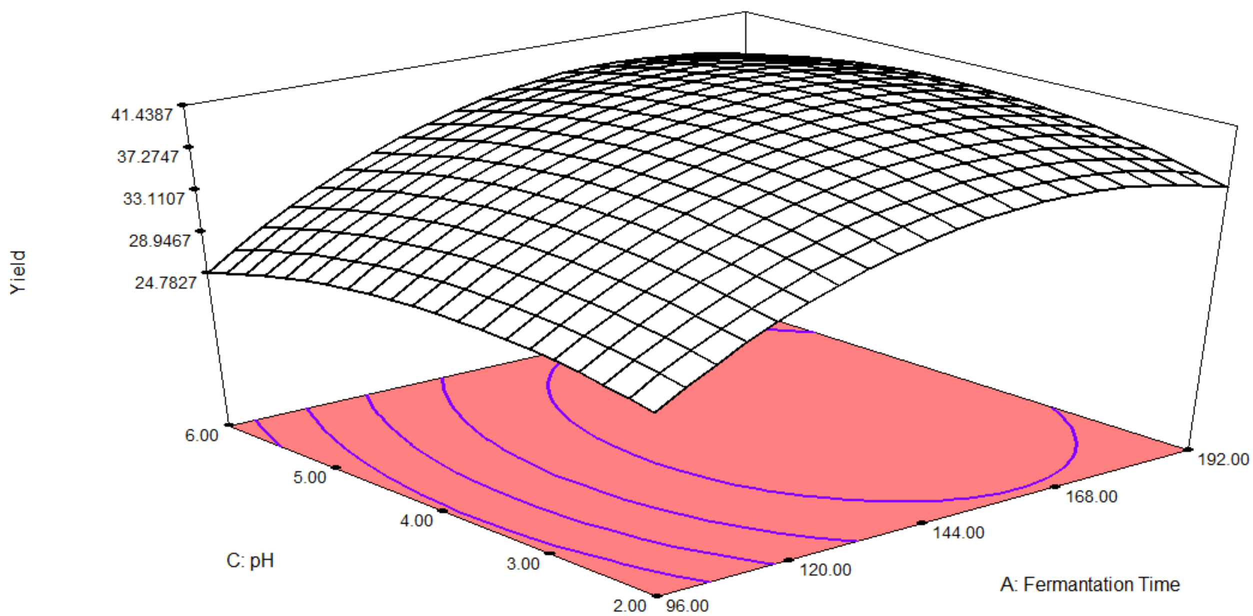


Figure 4.5 (a) 3D plot and (b) contour plot Interaction effect of fermentation time versus temperature at 4 pH.

4.7.2 Effects of fermentation period and pH on citric acid yield

The effects of fermentation time and pH on the response yield of extract by fixing the temperature at its center point was shown in the form of 3D plots and surface contours as illustrated in the Figure 4.6. As fermentation time and pH rise to center point the yield of citric acid increased but above the center point the yield becomes decline. As fermentation period increases further, to the center point more citric acid could be extracted which leads to the decline of the pH of the fermented broth to acidity. This creates acidic environment to the fungi which inhibited its growth and decrease in productivity might be due to decay in enzyme system responsible for citric acid biosynthesis.



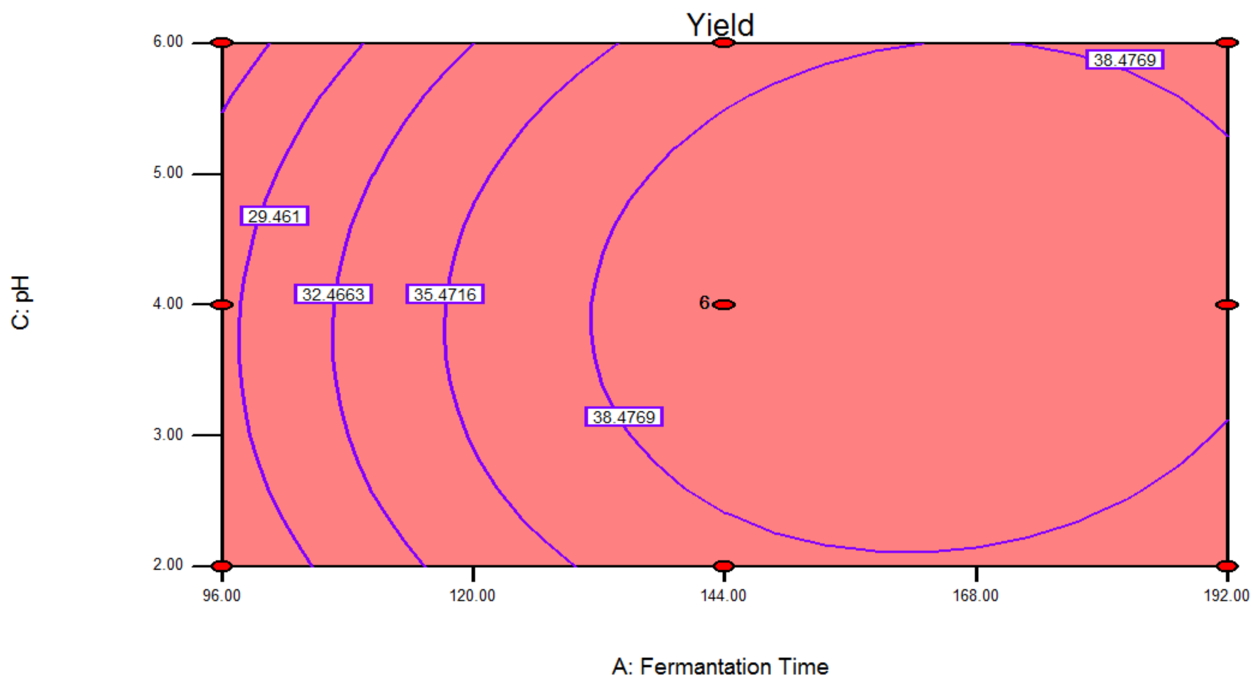


Figure 4.6 (a) 3D plot and (b) contour plot Interaction effect of fermentation time versus pH.

4.7.3 Effects of temperature and pH on citric acid yield

As it can be observed from Figure 4.7, moderate temperature and pH was the fermentation condition that results in maximum citric acid yield. When temperature and pH increases from low to moderate citric acid yield also increases. At higher temperature and pH, the yield become decreased. Because, further increasing of temperature denature the enzyme and retarded germination of fungal spores, slow metabolic activity, enzyme denaturation and low cell viability due to this the yield of citric acid decreased at higher temperature as indicated in Figure 4.7.

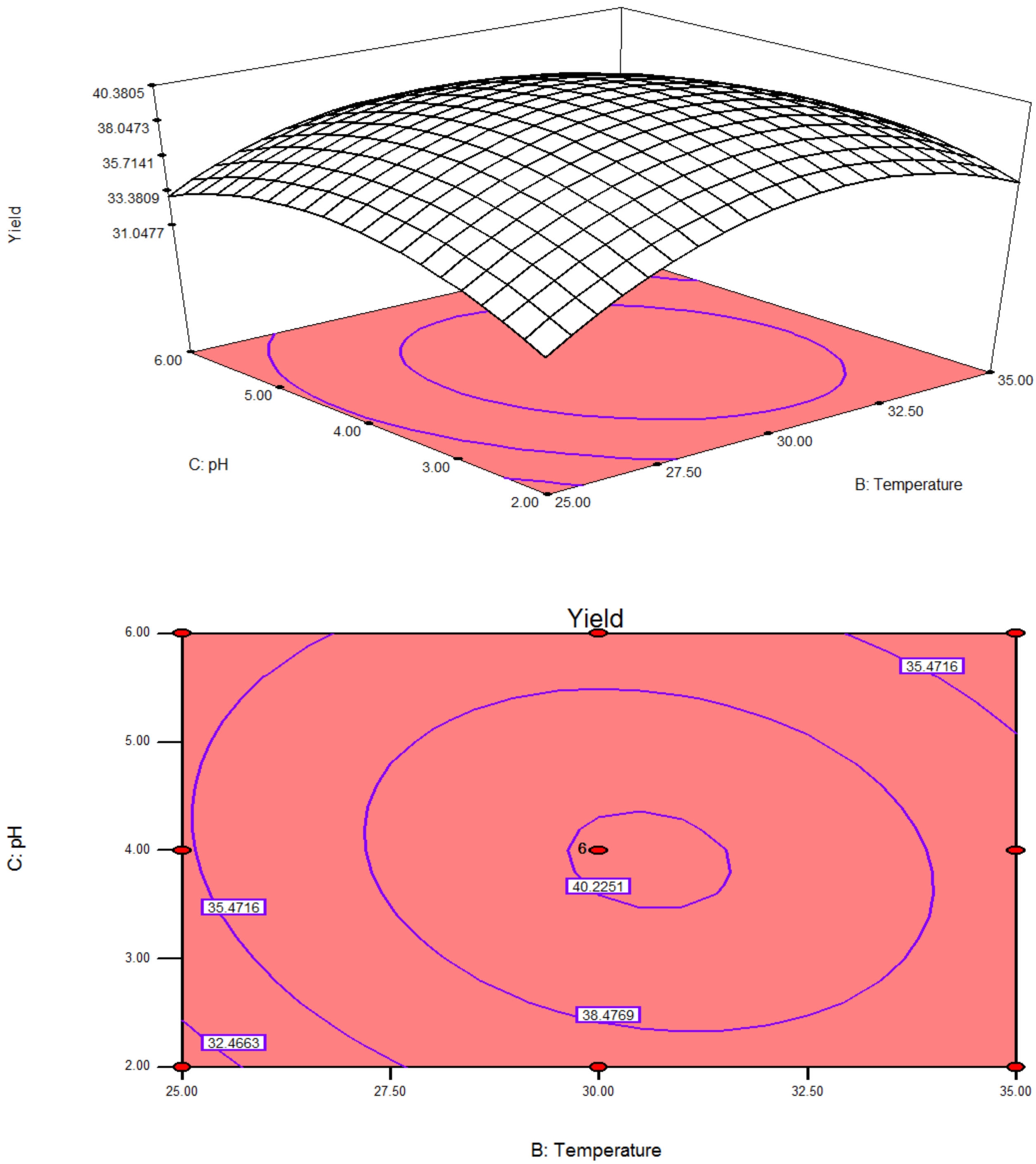


Figure 4.7 (a) 3D plot and (b) contour plot Interaction effect of fermentation temperature versus pH

4.8 Experimental optimization

One of the objectives of the present study was to find the optimal process parameters for better citric acid yield at minimum process economy. The process variables such as temperature, fermentation time and pH have been optimized. In optimizing the fermentation process, temperature, fermentation time and pH are a set of process parameters held to be "in range" while the citric acid yield was the response that need to be "maximized". Table 4.5 shows the summary of factors/responses and goals and the corresponding set of specific objectives that would optimize the process condition to have the highest responses. The expert design gives three different optimization choices, numerical optimization (set goal for each response), graphical optimization (set minimum and maximum limits for each response and then create an overlay highlighting an area of operability) and point prediction optimization (enter desired operating conditions and discover predicted response values with confidence intervals). In numerical optimization choice, depending on constraints (criteria) selected, different alternative solutions of optimization was given by expert design. For this study, numerical optimization was selected to obtain better highest response yield of the extracts.

Table 4.5 Constraints applied for optimization

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
Fermentation time(h)	is in range	96	192	1	1	3
Temperature(℃)	is in range	25	35	1	1	3
pH	is in range	2	6	1	1	3
Yield (g/L)	maximize	20.23	40.98	1	1	3

The expert design under numerical optimization gave 32 different alternative optimizing solutions. Depending on the parameters by compromising yield, economy and energy carrying, the best solution was selected among alternatives. Under taking the consideration of these constraints, 30.66℃, 3.89 pH and 156.00h were selected which gave 41.2963 g/L yield among optimizing alternatives given by expert design software. Desirability function was used to identify the optimum level of factors and to get maximum desirable responses. The optimized batch was selected with maximum combined desirability value i.e. 1.00 and the graph of the optimized solution for the fermentation condition was shown in figure 4.8.

Table 4.6 Different alternative optimization solutions

Number	Fermentation Time	Temperature	pH.	Yield	Desirability	
1	159.48	29.84	3.58	41.1782	1.000	No selected
2	166.24	29.11	4.51	41.0527	1.000	No selected
3	167.22	31.38	3.93	41.3288	1.000	No selected
4	171.62	30.89	4.00	41.3095	1.000	No selected
5	170.37	31.32	4.49	41.077	1.000	No selected
6	172.75	30.16	4.36	41.2221	1.000	No selected
7	156.00	30.66	3.89	41.2963	1.000	Selected
8	163.45	31.28	3.93	41.3831	1.000	No selected
9	161.33	31.42	4.31	41.2191	1.000	No selected
10	157.83	30.84	3.74	41.3178	1.000	No selected
11	171.67	30.94	4.12	41.2941	1.000	No selected
12	154.21	30.95	3.43	40.9853	1.000	No selected
13	174.29	31.38	4.29	41.0139	1.000	No selected
14	163.72	29.80	4.32	41.3549	1.000	No selected
15	165.41	29.90	4.39	41.3461	1.000	No selected

16	175.41	30.83	4.48	40.9891	1.000	No selected
17	159.29	30.62	4.65	41.0803	1.000	No selected
18	167.64	30.68	3.47	41.2059	1.000	No selected
19	168.97	31.35	3.87	41.2912	1.000	No selected
20	165.11	29.95	3.95	41.4244	1.000	No selected
21	171.32	29.13	4.42	41.0086	1.000	No selected
22	157.61	30.95	4.01	41.3355	1.000	No selected
23	161.61	29.07	4.15	41.1274	1.000	No selected
24	168.33	30.31	4.70	41.1182	1.000	No selected
25	162.97	31.29	3.35	41.0945	1.000	No selected
26	163.83	29.29	4.44	41.1601	1.000	No selected
27	160.52	32.03	4.01	41.0631	1.000	No selected
28	164.10	29.70	4.08	41.3817	1.000	No selected
29	159.29	30.60	3.33	41.0821	1.000	No selected
30	157.67	30.41	4.34	41.2741	1.000	No selected
31	170.88	31.48	4.37	41.0844	1.000	No selected
32	169.44	30.39	3.82	41.3661	1.000	No selected

Therefore, the numerical optimization can be taken as optimal value because the predicted value is close enough with actual value. The histogram shows how well each variables satisfied the criteria and values near one are good.

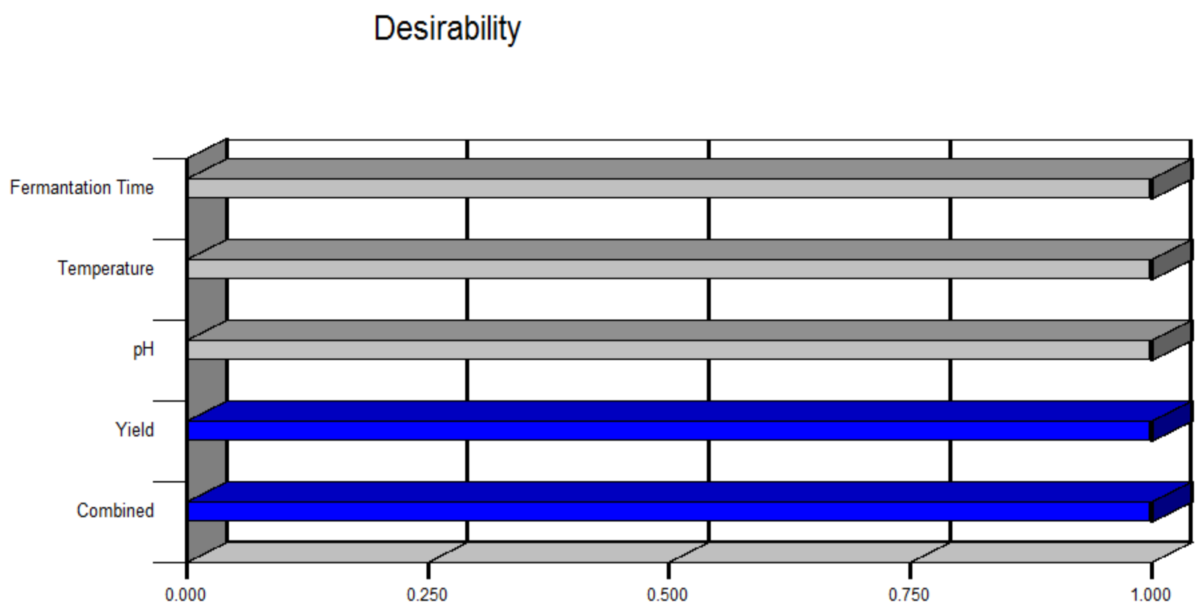


Figure 4.8 Desirability plot of optimization solution for the responses

The ramp plot of the optimized solution for the fermentation condition was shown in figure 4.9. The ramp display combines the individual graphs for easier interpretation and the dot on each ramp reflects the factor setting or response prediction for that solution.

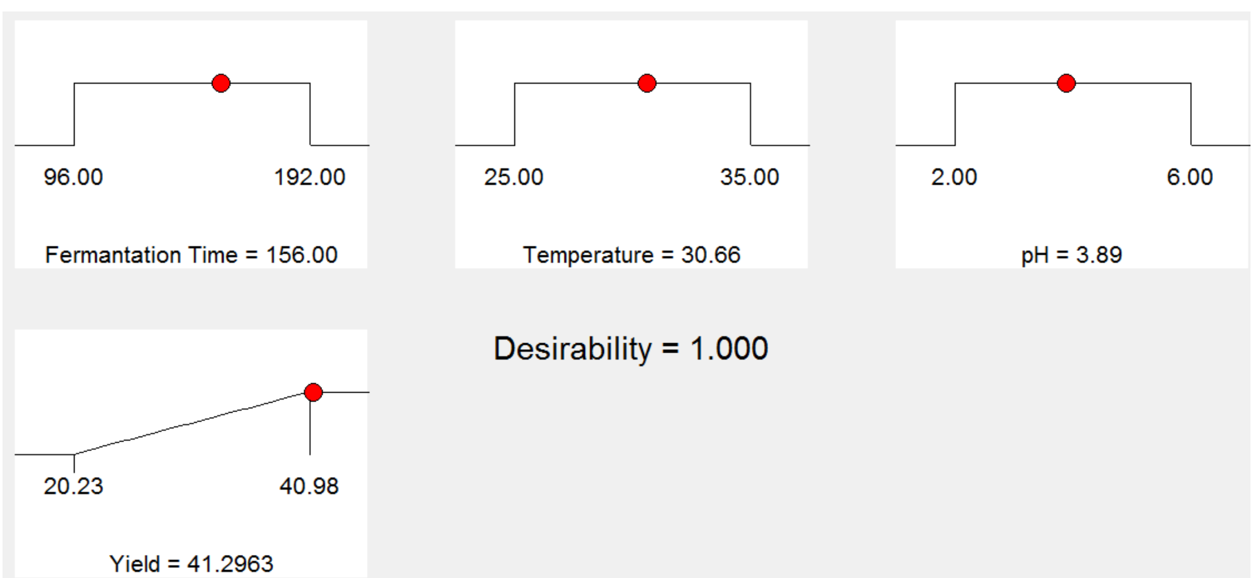


Figure 4.9 Ramp plot of optimization solution for the responses

The predicted highest screened yield was produced from solution of optimization at the moderate temperature (156h), moderate pH (3.89) and low fermentation time where the screened yield was at 41.29g/L as summarized in table 4.7. In order to verify this prediction, experiments were conducted and the results were computable with the prediction. This is good in terms of cost efficiency for commercial scale, as only small amount of energy power and moderate chemicals are needed for the quality citric acid production.

Table 4.7 Summary for optimization conditions

Incubation period	Temperature	pH	citric acid yield	
156 h	30 ℃	3.89	Predicted	Experimental
			41.29g/L	41.08g/L

4.9 Characterization and comparison of the product quality with standard

After optimization of the fermentation condition, citric acid was extracted from molasses at optimized fermentation condition as per the method presented in section 3.7. The obtained results were presented in Table 4.8 and compared with the standard as follows.

4.9.1 Sulphated ash

The sulfated ash test utilizes a procedure to measure the amount of residual substance not volatilized from a sample when the sample is ignited in the presence of sulfuric acid. The test is usually used for determining the content of inorganic impurities in an organic substance.

Total Sulphated ash contents of the product was calculated by direct substitution in the relationship expressed in Equation 3.10. The result of present study in terms of Sulphated ash contents was satisfactory as compared to the standard IS 13186 and citrique Belge as mentioned in the Table 4.8. The Sulphated ash content of the extracted citric acid in this study was lower than the standard which is 0.03 ppm <0.05 ppm. The amount of residue so obtained was not exceeds the limit specified in the standard. This implies that the product has good quality when compared to the standard in terms of the Sulphated ash.

4.9.2 Iron

Iron is one of the essential element that is required in trace amounts for various physiological processes especially for human being at recommended dose, but intake of this element at higher concentrations, above the standard may tend to be toxic and derange various physiological processes leading there by to diseases. Therefore, it is important to determine the metal concentrations in the extracted citric acid; this is because the product may use as food grade and added to various food items in the industries and consumed directly by human being. If its concentration is higher than specified standard it directly affect the human healthy.

In this study, as can be seen from table 4.8 results of the Iron content of the extracted citric acid was 2.2 ppm as assessed by Atomic Absorption Spectroscopy (Buck of Scientific 210 VGP AAS, USA), gave lower Iron content than IS 13186 Standard (i.e. 2.2 ppm< 5 ppm) but higher than Citrique Belge which is one of the greatest citric acid producer in the world (i.e. 2.2 ppm > 1 ppm) this may arise from technology difference specially the instrument and purification mechanism.

Figure 4.10 Detection of Iron content by Atomic Absorption Spectroscopy

Table 4.8 Physical and chemical properties of citric acid produced at optimized fermentation condition

Properties	**Present Results**	**IS 13186,2010 Standard**	**Citrique Belge**
Assay (%)	99.2	99.5	99.8 – 100.2
Iron (ppm)	2.2	5ppm max	< 1ppm
Arsenic(ppm)	BDL(<0.08)	1ppm max	< 1ppm
Lead(ppm)	BDL(<0.03)	3ppm max	< 0.5ppm
Sulphated Ash (%)	0.03	0.05ppm max	0.05%

4.9.3 Arsenic

Arsenic is odorless and tasteless. Inorganic arsenic is a known carcinogen and can cause cancer of the skin, lungs, liver and bladder. Lower level exposure can cause nausea and vomiting, decreased production of red and white blood cells, abnormal heart rhythm, damage to blood vessels, and a sensation of "pins and needles" in hands and feet. Therefore, in order to overcame the problem caused by intake of this heavy metal the quality of the product was assessed by Atomic Absorption Spectroscopy (Buck of Scientific 210 VGP AAS, USA). The results of elemental analysis in this study showed that the concentration of Arsenic in the sample was below the value specified in the standard (i.e. < 1 ppm for both IS 13186 and Citrique Belge Standard). The value indicated by the instrument was below detection limit of the instrument (i.e. < 0.08 ppm).

Figure 4.11 Detection of Arsenic content by Atomic Absorption Spectroscopy

4.9.4 Lead

Lead is one of the limited classes of element that can be described as purely toxic. Most other elements thought toxic and high concentration are actually required nutrient at lower levels. There is no exposure level below which lead appears to be safe. High level of lead is particularly of great concern especially due to the fact that citric acid was consumed by human who are uniquely susceptible to the effect of lead. As the result in table 4.8 showed the maximum amount of Lead in the sample was significantly lower than that of the standard.

Generally, this study shows that most of the quality of citric acid produced from molasses were in the acceptable value as compared to that of IS 13186 and Citrique Belge except assay (purity) of the product which was 99.2 %. This may arise from different factors like purification, for example Citrique Belge use ion exchange for further purification which was not used in this study. Therefore, keeping in view the future requirements and availability of cheap raw materials in the country, it would be worthwhile to develop and promote the process of citric acid fermentation, using locally discarded substrates such as molasses which otherwise are discharged to the river and causing environmental hazards.

5 CONCLUSIONS AND RECOMMENDATION

5.1 Conclusions

In this research, the utilization of molasses for citric acid production was investigated. The effects of fermentation parameters (i.e. fermentation time, temperature, and pH) on the citric acid yield have been studied. The outputs of the experiments have been analyzed by employing Design-Expert 6.0.8, three-level-three-factor and Response Surface Methods (RSM) through analysis of quality performance evaluation of characteristics parameter determination. Based on the experimental results obtained, it was found that all the process variables revealed significant interaction effect on citric acid yield result except the interaction between temperature and time.

According to the model regression equation developed, extraction yield was positively affected by linear effects except pH and negatively affected by all quadratic effects. In this study based on the analysis of experimental results, it is realized that the individual factors and their interaction effects are significant model terms on citric acid yield. This shows that the capability of the design of the experimental analysis is successfully capturing these effects. Optimal values of extraction conditions which gave maximum yield were selected using numerical optimization of design expert. The maximum citric acid yield obtained after conducting the experiment at selected optimum condition was 41.08g/L i.e. at the fermentation time of (156h), temperature of (30℃) and the pH of (3.89) with high value of combined desirability, i.e. 1. These values were selected from 32 alternative optimal solutions set by design expert, based on consider and compromise of yield, energy, and economy during extraction.

The observed quantitative difference in the quantity of the citric acid yield was due to pH, fermentation temperature and fermentation time variability. Thus, determination of the appropriate amount of the pH, optimal fermentation temperature and fermentation time needs to have a consideration to get the maximum amount of the required product. Based on the studies conducted, it was found that the properties of citric acid produced at the optimum condition (156h of fermentation time, 30℃ of fermentation temperature and 3.89 pH) were assessed by using Atomic Absorption Spectroscopy and the result were: content 99.2%, Sulphated ash 0.03 ppm, Iron content 2.2 ppm and, lead and Arsenic below detection limit (i.e. < 0.3 ppm and < 0.03 ppm). It can be concluded from these findings that comparable good quality citric acid can be produced from molasses fermentation with more environmentally friendly fermentation process.

Finally, it can be concluded that utilization of waste molasses for citric acid production is not only solve the problem of waste disposal but also save valuable foreign exchange by reducing the citric acid imported from abroad.

5.2 Recommendation

Given the demonstrated possibility of utilizing molasses for citric acid production therefore; further works are still necessary. Future researches that would be complementary to the present study aiming at investigation of the better citric acid yield and concerning the environment, therefore, strongly recommended to focus on the following points:

- Experiments performed on fermentation of molasses, showed that good quality grade citric acid can be produced from molasses. But before implementing further studies; process design and feasibility studies need to be carried out.
- One can also investigate the effects of low grade alcohols such as ethanol, methanol on a citric acid yield.
- Storage techniques of molasses could also be studied for production of citric acid.
- Investigation on the elemental analysis of the molasses must be conducted.
- Further studies are needed in various topics related to utilization and best incorporation techniques of other waste materials in citric acid production.
- It is recommended to conduct researches to measure the effects of sugar concentration on citric acid yield.
- It is recommended to conduct researches on purification using ion exchange.
- Further studies are needed to determine concentration of citric acid by using High-performance Liquid Chromatograph.
- Further investigation is needed to identify the product is either monohydrate or anhydrous.

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APPENDICES

Appendix A Experimental Design and Analysis Data

Table A1 Estimated model equation coefficients

Factors	Coefficient Estimate	DF	Standard Error	95% CI Low	95% CI High	VIF
Intercept	40.33	1	0.18	39.95	40.71	1.00
A-Fermentation Time	5.27	1	0.13	5.00	5.54	1.00
B-Temperature	0.95	1	0.13	0.68	1.22	1.00
C-pH	-0.14	1	0.13	-0.41	0.13	1.10
A2	-6.23	1	0.20	-6.65	-5.80	1.10
B2	-4.20	1	0.20	-4.62	-3.77	1.10
C2	-3.12	1	0.20	-3.55	-2.70	1.00
AB	-0.23	1	0.16	-0.56	0.094	1.00
AC	0.78	1	0.16	0.46	1.11	1.00
BC	-1.15	1	0.16	-1.48	-0.82	1.00

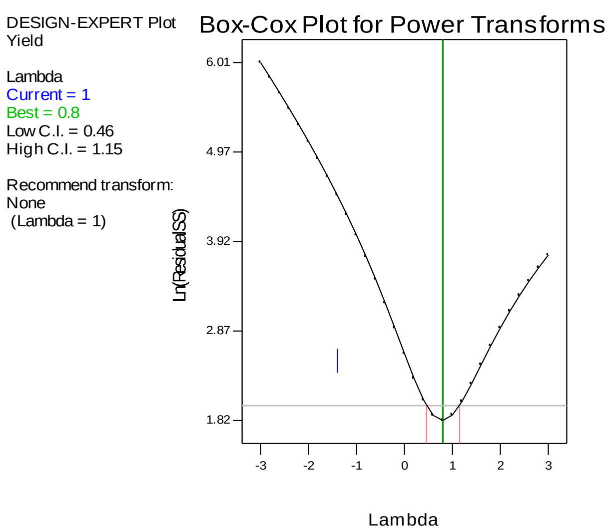

Figure A3 Plot of Box-Cox for power transform and (b) Studentized Residuals

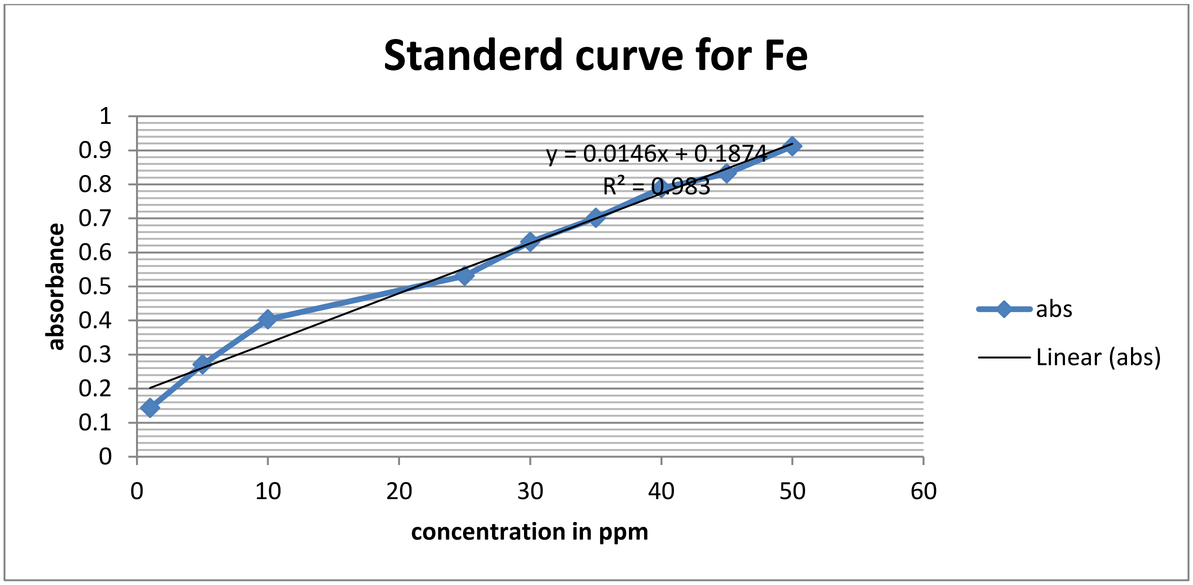

Figure A4 Standard curve for Iron

Appendix B Diagrams and Photos

(a) (b) (c)

Figure B1) (a) *A.nigures* before incubation (b) Laboratory incubator and (c) Cultured *A.nigures* after 5days

Figure B2) (a) Atomic Absorption Spectroscopy

Figure B3) (a) Samples ready for crystallization

Declaration

I, undersigned, declare that the thesis for the M.Sc. degree in Process Engineering at the University of Addis Ababa, hereby submitted by me, is my original work and has not previously been submitted for degree at this or any other university, and that all resources of materials used for this thesis have been duly acknowledged.

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Signature: ---

Date of Submission: --------------------------------------

This thesis has been submitted for examination with my approval as a university advisor.

Name: Dr. Anuradha Jabasingh

Signature: --------------------------------

Date: -------------------------