**Study material**

**B.Sc. (H) Botany III**

**DSE: IEM theory**

**Unit 3**

The book of **Principles of Fermentation Technology** by Stanbury PF, Whitaker A, Hall SJ, Second edition, Elsevier, can be referred to **for** the following topics-

1. Centrifugation with types- Basket, Tubular-bowl, sold-bowl scroll (de-canter), multi-chamber, disc-bowl
2. Cell disruption with types- Physico (p)-mechanical (m): liquid shear (m), sold shear (m), agitation with abrasives (m), freezing thawing (p), decompression (p), ultrasonication (m) and Chemical: solvents, detergents, osmotic shock, alkali treatment, and Enzyme treatment
3. Solvent extraction- liquid- liquid extraction with distribution or partition coefficient and types of single -stage, multi-stage (co-current and countercurrent systems), two phase aqueous extraction, supercritical fluid extraction
4. Drying and types- contact dries, spry drier, freeze drying with lyophilization

**Production and estimation of**

Enzyme- **any one**- Amylase or lipase

Organic acid- **any one**- citric acid or glutamic acid

Alcohol- ethanol

Antibiotic- penicillin

**Enzyme- Amylase- Reference 1**

<https://www.hindawi.com/journals/bmri/2017/1272193/>

Biologically active enzymes can be obtained from plants, animals, and microorganisms. Microbial enzymes have been generally favored for their easier isolation in high amounts, low-cost production in a short time, and stability at various extreme conditions, and their cocompounds are also more controllable and less harmful. Microbially produced enzymes that are secreted into the media are highly reliable for industrial processes and applications. Furthermore, the production and expression of recombinant enzymes are also easier with microbes as the host cell.

Amylases are broadly classified into α, β, and γ subtypes, of which the first two have been the most widely studied. α-Amylase is a faster-acting enzyme than β-amylase. The amylases act on α-1-4 glycosidic bonds and are therefore also called glycoside hydrolases. Amylases are distributed widely in living systems and have specific substrates. Amylase substrates are widely available from cheap plant sources, rendering the potential applications of the enzyme more plentiful in terms of costs. Amylases can be divided into endoamylases and exoamylases. The endoamylases catalyze hydrolysis in a random manner within the starch molecule. This action causes the formation of linear and branched oligosaccharides of various chain lengths. The exoamylases hydrolyze the substrate from the nonreducing end, resulting in successively shorter end products. All α-amylases (EC 3.2.1.1) act on starch (polysaccharide) as the main substrate and yield small units of glucose (monosaccharide) and maltose (disaccharide). Starch is made up of two glycose polymers, amylose and amylopectin, which comprise glucose molecules that are connected by glyosidic bonds. Both polymers have different structures and properties. A linear polymer of amylose has a maximum of 6000 glucose units linked by α-1,4 glycosidic bonds, whereas amylopectin is composed of α-1,4-linked chains of 10–60 glucose units with α-1,6-linked side chains of 15–45 glucose units. The α-amylases are metalloenzymes that require metal (calcium) ions to maintain their stability, activity, and structural confirmation. Based on sequence alignments of α-amylases, these enzymes have four conserved arrangements (I–IV), which are found as β-strands 3, 4, and 5 in the loop connecting β-strand 7 to α-helix 7.

Industrial Applications of Microbial Amylase

Amylase makes up approximately 25% of the world enzyme market. It is used in foods, detergents, pharmaceuticals, and the paper and textile industries. Its applications in the food industry include the production of corn syrups, maltose syrups, glucose syrups, and juices and alcohol fermentation and baking. It has been used as a food additive and for making detergents. Amylases also play an important role in beer and liquor brewing from sugars (based on starch). In this fermentation process, yeast is used to ingest sugars, and alcohol is produced. Fermentation is suitable for microbial amylase production under moisture and proper growth conditions. Two kinds of fermentation processes have been followed: submerged fermentation and solid-state fermentation. The former is the one traditionally used and the latter has been more recently developed. In traditional beer brewing, malted barley is mashed and its starch is hydrolyzed into sugars by amylase at an appropriate temperature. By varying the temperatures and conditions for α- or β-amylase activities, the unfermentable and fermentable sugars are determined. With these changes, the alcohol content and flavor and mouthfeel of the end product can be varied.

Isolation Methods

The isolation of potential and efficient bacterial or fungal strains is important before being screened for their production of enzymes of interest. Microbes are ubiquitous and can be obtained from any source. However, the most efficient strains are usually obtained from substrate-rich environments, from which the microbes can be adopted to use a particular substrate. The common method of strain isolation is through serial dilution, whereupon the number of colonies is minimized and thus easy to select. Another method is through substrate selection, where efficient strains are isolated according to their affinity for a particular substrate. Through these methods, several bacteria and fungi have been isolated and studied for amylase production.

Microbial Amylase

Microbial amylases obtained from bacteria, fungi, and yeast have been used predominantly in industrial sectors and scientific research. The level of amylase production varies from one microbe to another, even among the same genus, species, and strain. Furthermore, the level of amylase production also differs depending on the microbe’s origin, where strains isolated from starch- or amylose-rich environments naturally produce higher amounts of enzyme. Factors such as pH, temperature, and carbon and nitrogen sources also play vital roles in the rate of amylase production, particularly in fermentation processes. Because microorganisms are amenable to genetic engineering, strains can be improved for obtaining higher amylase yields. Microbes can also be fine-tuned to produce efficient amylases that are thermostable and stable at stringent conditions. Such improvements can also reduce contamination by background proteins and minimize the reaction time and lead to less energy expenditure in the amylase reaction. The selection of halophilic strains is also beneficial to the production of amylase under extreme conditions.

Bacterial Amylases

Among the wide range of microbial species that secrete amylase, its production from bacteria is cheaper and faster than from other microorganisms. Furthermore, as mentioned above, genetic engineering studies are easier to perform with bacteria and they are also highly amenable for the production of recombinant enzymes. A wide range of bacterial species has been isolated for amylase secretion. Most are *Bacillus* species (*B. subtilis, B. stearothermophilus, B. amyloliquefaciens, B. licheniformis, B. coagulans, B. polymyxa, B. mesentericus, B. vulgaris, B. megaterium, B. cereus, B. halodurans,* and *Bacillus sp. Ferdowsicous*), but amylases from *Rhodothermus marinus, Corynebacterium gigantea, Chromohalobacter sp., Caldimonas taiwanensis, Geobacillus thermoleovorans, Lactobacillus fermentum, Lactobacillus manihotivorans,* and *Pseudomonas stutzeri* have also been isolated. Halophilic strains that produce amylases include *Haloarcula hispanica, Halobacillus sp., Chromohalobacter sp., Bacillus dipsosauri,* and *Halomonas meridiana*.

Fungal Amylases

Fungal enzymes have the advantage of being secreted extracellularly. In addition, the ability of fungi to penetrate hard substrates facilitates the hydrolysis process. In addition, fungal species are highly suitable for solid-based fermentation. Efficient amylase-producing species include those of genus *Aspergillus* (*A. oryzae, A. niger, A. awamori, A. fumigatus, A. kawachii,* and *A. flavus*), as well as *Penicillium* species (*P. brunneum, P. fellutanum, P. expansum, P. chrysogenum, P. roqueforti, P. janthinellum, P. camemberti,* and *P. olsonii*), *Streptomyces rimosus, Thermomyces lanuginosus, Pycnoporus sanguineus, Cryptococcus flavus, Thermomonospora curvata,* and *Mucor* sp.

Estimation- Screening Microbial Amylase Production

Production or secretion of amylase can be screened by different common methods, including solid-based or solution-based techniques. The solid-based method is carried out on nutrient agar plates containing starch as the substrate, whereas solution-based methods include the dinitro salicylic acid (DNS) and Nelson-Somogyi (NS) techniques.

In the solid-agar method, the appropriate strain (fungi or bacteria) is pinpoint-inoculated onto the starch-containing agar at the center of the Petri plate. After an appropriate incubation period, the plate is flooded with iodine solution, which reveals a dark bluish color on the substrate region and a clear region (due to hydrolysis) around the inoculum, indicating the utilization of starch by the microbial amylase.

In the solution-based DNS method, the appropriate substrate and enzyme are mixed in the right proportion and reacted for 5 min at 50°C. After cooling to room temperature, the absorbance of the solution is read at 540 nm. Similarly, in the NS method, amylase and starch are mixed and incubated for 5 min at 50°C. Then, a Somogyi copper reagent is added to stop the reaction, followed by boiling for 40 min and a subsequent cool-down period. A Nelson arsenomolybdate reagent is then added and the mixture is incubated at room temperature for 10 min. Then, after diluting with water, the solution is centrifuged at high speed and the supernatant is measured at 610 nm. Apart from these, several other methods are available for amylase screening, but all use the same substrate (starch).

**Reference 2**

<http://pubs.sciepub.com/jaem/2/4/10/>

Types of Amylase

α-Amylase

α-Amylase (E.C.3.2.1.1) is a hydrolase enzyme that catalyses the hydrolysis of internal α-1, 4-glycosidic linkages in starch to yield products like glucose and maltose. It is a calcium metalloenzyme i.e. it depends on the presence of a metal co factor for its activity. There are 2 types of hydrolases: endo-hydrolase and exo-hydrolase. Endo- hydrolases act on the interior of the substrate molecule, whereas exo-hydrolases act on the terminal non reducing ends. Hence, terminal glucose residues and α-1, 6-linkages cannot be cleaved by α-amylase. The substrate that α-amylase acts upon is starch. Starch is a polysaccharide composed of two types of polymers – amylose and amylopectin. Amylose constitutes 20-25% of the starch molecule. It is a linear chain consisting of repetitive glucose units linked by α-1, 4-glycosidic linkage. Amylopectin constitutes 75-80% of starch and is characterized by branched chains of glucose units. The linear successive glucose units are linked by α-1, 4-glycosidic linkage while branching occurs every 15-45 glucose units where α-1, 6 glycosidic bonds are present. The hydrolysate composition obtained after hydrolysis of starch is highly dependent on the effect of temperature, the conditions of hydrolysis and the origin of enzyme. The optimum pH for activity is found to be 7.0.α-Amylase has become an enzyme of crucial importance due to its starch hydrolysis activity and the activities that can be carried out owing to the hydrolysis. One such activity is the production of glucose and fructose syrup from starch. α-Amylase catalyses the first step in this process. Previously, starch was hydrolyzed into glucose by acid hydrolysis. But this method has drawbacks like the operating conditions are of highly acidic nature and high temperatures. These limitations are overcome by enzyme hydrolysis of starch to yield high fructose syrup. The use of enzymes in detergents formulations has also increased dramatically with growing awareness about environment protection. Enzymes are environmentally safe and enhance the detergents ability to remove tough stains. They are biodegradable and work at milder conditions than chemical catalysts and hence preferred to the latter.

β-Amylase

β-Amylase (EC 3.2.1.2) is an exo-hydrolase enzyme that acts from the nonreducing end of a polysaccharide chain by hydrolysis of α-1, 4-glucan linkages to yield successive maltose units. Since it is unable to cleave branched linkages in branched polysaccharides such as glycogen or amylopectin, the hydrolysis is incomplete and dextrin units remain. Primary sources of β-Amylase are the seeds of higher plants and sweet potatoes. During ripening of fruits, β-Amylase breaks down starch into maltose resulting in the sweetness of ripened fruit. The optimal pH of the enzyme ranges from 4.0 to 5.5. β-Amylase can be used for different applications on the research as well as industrial front. It can be used for structural studies of starch and glycogen molecules produced by various methods. In the industry it is used for fermentation in brewing and distilling industry. Also, it is used to produce high maltose syrups.

γ-Amylase

γ-Amylase (EC 3.2.1.3 ) cleaves α(1-6)glycosidic linkages, in addition to cleaving the last α(1-4)glycosidic linkages at the nonreducing end of amylose and amylopectin, unlike the other forms of amylase, yielding glucose. γ- amylase is most efficient in acidic environments and has an optimum pH of 3.

**Production of α-Amylase**

Sources

α-Amylase can be isolated from plants, animals or microorganisms. The enzyme has been isolated from barley and rice plants. It has been found that cassava mash waste water is a source of α-Amylase which is active in wide range of pH and temperature. In the recent past, there has been extensive research on microbial production of α-Amylase. There are 2 major reasons for the increasing interest in microbial sources:

1. The growth of microorganisms is rapid and this will in turn speed up the production of enzyme. Microorganisms are easy to handle when compared to animals and plants. They require lesser space and serve as more cost effective sources.
2. Microorganisms can be easily manipulated using genetic engineering or other means. They can be subjected to strain improvement, mutations and other such changes by which the production of α-Amylase can be optimized. Also, the microorganisms can be tailored to cater to the needs of growing industries and to obtain enzymes with desired characteristics like thermostability for example. Thermostable α-Amylases are desired as they minimize contamination risk and reduce reaction time, thus saving considerable amount of energy. Also when hydrolysis is carried out at higher temperatures, the polymerization of D-glucose to iso-maltose is minimized.

α-Amylase is produced by several bacteria, fungi and genetically modified species of microbes. The most widely used source among the bacterial species is the Bacillus spp. B. amyloliquefaciens and B. licheniformis are widely used for commercial production of the enzyme. Other species which have been explored for production of the enzyme include B.cereus and B. subtilis to name a few. α-Amylases produced from Bacillus licheniformis, Bacillus stearothermophilus, and Bacillus amyloliquefaciens show promising potential in a number of industrial applications in processes such as food, fermentation, textiles and paper industries. Bacillus subtilis, Bacillus stearothermophilus, Bacillus licheniformis and Bacillus amyloliquefaciens are known to be good producers of thermostable α-Amylase. Thermostability is an important characteristic as enzymatic liquefaction and saccharification of starch are performed at high temperatures (100–110°C). Thermostable amylolytic enzymes are being investigated to improve industrial processes of starch degradation and are useful for the production of valuable products like glucose, crystalline dextrose, dextrose syrup, maltose and maltodextrins. Use of enzyme produced by thermophiles has the added advantage of reduced risk of contamination by mesophiles. Enzymes produced by some halophilic microorganisms are stable at high salinities and therefore could be used in many harsh industrial processes where the concentrated salt solutions are used. The halophilic nature the enzyme prevents inhibition of its activity under these conditions which would otherwise occur if a normal enzyme is used. In addition, most halobacterial enzymes are considerably tolerant to high temperatures and remain stable at room temperature over long periods. Halophilic amylases from halophilic bacteria such as Chromohalobacter sp., Halobacillus sp., Haloarcula hispanica, Halomonas meridiana, and Bacillus dipsosauri have been characterized.

Fungal sources of α-Amylase are confined to terrestrial isolates, mostly to Aspergillus species and to only few species of Penicillium, P. brunneum being one of them. Penicillium fellutanum has been used in the recent past to produce α-Amylase by submerged fermentation. Penicillium expansum MT-1 has been used to produce the enzyme by solid state fermentation. Here, Loquat (Eriobotrya japonica Lindley) kernels were used as the substrate for growth of the fungi. Penicillium chrysogenum was used as the microbial source for producing amylase by solid state fermentation using various substrates such as, corncob leaf, rye straw, wheat straw and wheat bran. The fungal source used predominantly for commercial production of α-Amylase are the strains of Aspergillus spp. Aspergillus oryzae, A. niger and A. awamori are most commonly used species for commercial production among several others. Aspergillus fumigatus has been employed for production of the enzyme by submerged fermentation technique.



**Production Methods**

There are mainly two methods which are used for production of α-Amylase on a commercial scale. These are: 1. Submerged fermentation and 2. Solid State fermentation. The latter is a fairly new method while the former is a traditional method of enzyme production from microbes which has been in use for a longer period of time.

Submerged fermentation (SmF) employs free flowing liquid substrates, such as molasses and broths. The products yielded in fermentation are secreted into the fermentation broth. The substrates are utilized quite rapidly; hence the substrates need to be constantly replenished. This fermentation technique is suitable for microorganisms such as bacteria that require high moisture content for their growth. SmF is primarily used for the extraction of secondary metabolites that need to be used in liquid form. This method has several advantages. SmF allows the utilization of genetically modified organisms to a greater extent than SSF. The sterilization of the medium and purification process of the end products can be done easily. Also the control of process parameters like temperature, pH, aeration, oxygen transfer and moisture can be done conveniently.

Solid state fermentation is a method used for microbes which require less moisture content for their growth. The solid substrates commonly used in this method are, bran, bagasse, and paper pulp. The main advantage is that nutrient-rich waste materials can be easily recycled and used as substrates in this method. Unlike SmF, in this fermentation technique, the substrates are utilized very slowly and steadily. Hence the same substrate can be used for a longer duration, thereby eliminating the need to constantly supply substrate to the process. Other advantages that SSF offers over SmF are simpler equipments, higher volumetric productivity, higher concentration of products and lesser effluent generation. For several such reasons SSF is considered as a promising method for commercial production of enzymes.

Fungal sources have been investigated for α-Amylase production through submerged and solid state fermentation. However, studies reveal that SSF is the most appropriate process in developing countries due to the advantages it offers which make it a cost effective production process. Also SSF provides a medium that resembles the natural habitat of fungal species, unlike Smf which is considered a violation of their habitat.

Process Parameters

The optimum process control parameters vary depending on the microbial source, desired end product, method of fermentation employed and many other such factors.

Temperature

There are two temperatures that need to be in optimum range during production. They are temperature for the growth of the microbial source and optimum temperature at which maximum production of enzyme takes place. The optimum temperatures for both were studied in strains of B. licheniformis and B. subtilis. The optimum temperatures for growth and α-Amylase production were found to be 45°C to 46 °C and 50 °C, respectively. In B. subtilis the optimum temperature for growth (42°C) was higher by 5°C than that of amylase formation (37°C). Thermophilic archaeal α-Amylases are active and grow at high temperatures. Studies by B. Jensen, et al. (1992) and L. Bunni,et al. (1989) reveal that optimal production of amylase is between 50–55°C for the thermophilic fungal cultures such as Talaromyces emersonii, Thermomonospora fusca and Thermomyces lanuginosus. Thermococcus α-Amylases are optimally active at temperatures close to 80°C. Pyrococcus enzymes are optimally active around 100°C. An optimum range of 95-100°C was observed to yield maximum enzyme activity in a study involving Pyrococcus furiosus. Penicillium fellutanum showed an optimum activity of 98±4.6 U/ml at a temperature of 30°C. An optimal temperature of 50oC was observed when temperature was varied within a range of 30-90°C for production of α-Amylase by Aspergillus oryzae. When solid state fermentation was carried out for production of the enzyme by Penicillium janthinellum in a range of 30-55°C, the optimum incubation temperature was found to be 45°C. The enzyme production increases with increase in temperature till it reaches the optimum. With further increase in temperature the enzyme production decreases. This may be due to the loss of moisture in the substrate which adversely affects the metabolic activities of the microbes leading to reduced growth and decline in enzyme production. The optimum temperature for production of the enzyme by Bacillus sp. isolated from dhal industry waste was found to be 60°C. The α-Amylase produced by Clostridium acetobutylicum was incubated at temperatures ranging from 30-60°C. The optimal activity was found to be at 45°C. Rhodothermus marinus, a thermophilic marine microorganism exhibited optimal yield of thermostable α-Amylase at 61°C. Maximum α-Amylase production of a-amylase by Alteromonas haloplanktis was found at an optimal temperature of 4°C.

pH

Optimum pH is a critical factor for the stability of enzyme produced. Enzymes are pH sensitive and hence care must be taken to control the pH of the production process. Pyrococcus furiosus produces α-Amylase which shows activity at an optimum pH of 6.5–7.5. The production of extracellular amylase by the thermophilic fungus Thermomyces lanuginosus was investigated in solid state fermentation (SSF). The maximum enzyme activity was 534 U/g when wheat bran was used as the substrate with optimum pH conditions of 6.0. Bacillus amyloliquefaciens produces the enzyme with an optimum pH of 7.0. Halomonas meridiana was studied for optimization of α-Amylase production. The study revealed that the amylase exhibited maximal activity at pH 7.0, being relatively stable in alkaline conditions. The optimum pH for production of the enzyme by Bacillus sp. isolated from dhal industry waste was found to be 6.5. Aspergillus sp.such as A. oryzae, A. ficuum and A. niger exhibit optimal production at pH 5.0–6.0 in SmF. Saccharomyces cerevisiae and S. kluyveri have shown maximum yield of enzyme at pH 5.0 and pH of 7.0 respectively. An initial pH range 7.5 to 8 was found to be optimal for enzyme production by Rhodothermus marinus. Thermophilic microorganisms such as P. woesei and Thermococcus profundus exhibited optimal production of α-Amylase at pH value of 5.0. Clostridium thermosulfurogenes yielded maximum enzyme at a pH of 7.0. An optimum pH of 9.0 was observed to yield enzyme with specific activity of 138 U/mg in the fungi Preussia minima. During production of α-Amylase from P. fellutanum the optimum pH was found to fall within the range of 6.0 to 7.0.

Duration of fermentation

This is a crucial factor in fermentation process. If the process is carried out for a time period shorter than the optimum duration the maximum yield cannot be obtained. The enzyme activity increases with increase in incubation time till it reaches the optimum duration. In most cases, the production of enzyme begins to decline if the incubation time is further increased. This could be due to the depletion of nutrients in the medium or release of toxic substances. Bacillus subtilis, a well known producer of alpha amylase was studied for comparison between different fermentation hours and the study revealed a high yield of alpha amylase after 48 hours of fermentation. Different incubation time durations were compared for yield of amylase from Penicillium fellutanum isolated from mangrove rhizosphere soil. The culture when incubated at 96 h, showed the maximum activity of 136 U/ ml. This was a 2 fold increase in activity when compared to 24 h incubation (68±2.3 U/ml). In an attempt to produce α-Amylase from A. oryzae using coconut oil cake as a substrate, the maximum activity was found to be 1752 U/gds which resulted after 72 h of incubation. An optimum α-Amylase activity of 62 470 U/g was found after fermentation for 72 hours when produced by Bacillus amyloliquefaciens.

Substrate

Carbon Source: Common carbon sources used as substrates include maltose, sucrose, starch and glucose. In the recent past various substrates have been investigated for use in SSF technique. The aim is to use substrates that are waste or by products of other processes in order to make the process of enzyme production environment friendly. One such substrate is oil cake. Oil cakes are byproducts of oil extraction. The chemical composition of oil cake depends upon the extraction methods used. Due to the advantages that SSF offers bacterial species have also been used to produce the enzyme under SSF conditions. Bacillus subtilis was studied for production of α-Amylase using solid state fermentation for 48 hours at 37°C with wheat bran as substrate. The specific activity of amylase was found to be 13.14 µmol/mg/min at 40°C. Wheat bran has been used as substrate for α-Amylase production by B.licheniformis and A.niger. Studies on screening various combinations of substrates for optimum yield of enzyme by B. amyloliquefaciens have shown that wheat bran and groundnut oil cake in a ratio of 1:1 yield enzyme with activity of 1 671 U/g. Banana waste has been used for production by B.subtilis. Investigation of production of α-Amylase by Bacillus cereus MTCC 1305 revealed that among different carbon sources that were studied, glucose (0.04 g/g) showed enhanced enzyme production (122±5) U/g). On the other hand supplementation of different nitrogen sources (0.02 g/g) showed decline in enzyme production.

Nitrogen Source: The nitrogen source used for production of α-Amylase may be organic or inorganic. Few of the inorganic nitrogen sources include ammonium sulphate, ammonium chloride and ammonium hydrogen phosphate. Most commonly used organic sources of nitrogen include peptone, yeast extract and soyabean meal. In a study of production of the enzyme by Aspergillus oryzae, NaNO3 (0.9%) and malt (1%) gave highest yield of enzyme among many other nitrogen sources. In another study of Aspergillus oryzae organic sources did not yield good results. Inorganic sources such as ammonium chloride, ammonium phosphate, ammonium sulphate resulted in low yield of enzyme. Only when the inorganic sources were replaced by ammonium nitrate and sodium nitrate did the yield increase marginally. In a study conducted on Penicillium fellutanum the maximum activity of 150 U /ml was obtained when peptone was used as the nitrogen source. This was about 9% higher than the activity obtained by using yeast, meat and casein. Peptone is usually the most high yielding nitrogen source when employed in solid- state fermentation. A maximum production of 812 U/g was achieved when peptone was employed as the nitrogen source for α-amylase production by P. expansum. Among the inorganic nitrogen sources, ammonium salts showed better results when compared to sodium nitrate. Studies reveal that peptone, sodium nitrate and casein hydrolysate are good nitrogen sources for A. fumigatus and A.niger. Among the nitrogen sources tested for Thermomyces lanuginosus, peptone showed the best results for enzyme production (414 U/g). Tryptone (356 U/g) and meat extract (338 U/g) also yielded good amount of enzyme.

Moisture: Optimum levels of initial moisture content may vary depending on the microbial source used. For fungal sources the moisture content required is less whereas bacterial sources need more moisture content for high yield of the enzyme. The optimum level can be determined by determining the enzyme yield within a range of initial moisture content. In a study of production of α-Amylase by Penicillium janthinellum the moisture content was varied within a range of 20- 80% by varying the amount of salt solution used in moistening the substrate particles. The study revealed that with wheat bran as the substrate, the maximum yield of enzyme was of 295 U/gds with 60% of initial moisture content. The yield decreased to 220 U/gds at 80% (w/w) moisture content. For Aspergillus oryzae the maximum yield was found to be 9000 U/gds at an initial moisture content of 60% which declined drastically when the moisture content was increased to 70%. The optimal initial moisture content may also vary with substrate in addition to the microbial source used. This may be due to fact that substrates with better water holding capacity require lower moisture content for optimal production of the enzyme when compared to others. Production of α-Amylase from Penicillium chrysogenum was carried out using various agricultural by- products like wheat bran (WB), corncob leaf (CL), rye straw (RS) and wheat straw (WS). Enzyme yields were 20, 34, 40 and 127 U/mL with CL, RS, WS and WB at 75, 55, 65 and 65% of initial moisture content, respectively. For B. amyloliquefaciens, studies were carried out to optimize the process parameters in which the initial moisture content was varied from 55- 90% for optimization. An optimal production of 3677 U/g of α-Amylase was found at 85% of initial moisture content using a combination of wheat bran and groundnut oil cake as the substrate. The enzyme yield usually increases with increase in initial moisture content reaching an optimal level followed by decrease in enzyme yield with further increase in moisture content. At low moisture content there is high water tension and low solubility of the nutrients which causes the low yield of enzyme. With increase in moisture content the swelling of substrate takes place which ensures better uptake of nutrients by the microbes. But with increase of moisture content further the enzyme yield decreases owing to many reasons. The inter particle distances reduce and can result in agglomeration of substrate particles. Also the reduction in gas volume and gas diffusion results in impaired oxygen transfer.

Purification of α-Amylase

Enzymes used for industrial applications are usually crude preparations and require less downstream processing. Whereas the enzymes used clinical and pharmaceutical industry need to be highly purified. Also when used for study of structure function relationships and biochemical properties the enzymes have to be in purified form. Purification methods commonly employed are precipitation, chromatography and liquid-liquid extraction depending on the properties of the enzyme desired. A combination of the above methods is used in a series of steps to achieve high purity. The number of steps involved in purification will depend on the extent of purity that is desired. The crude extracellular enzyme sample can be obtained from the fermented mass by filtration and centrifugation. In the case of intracellular enzymes, raw corn starch may be added followed by filtration and subsequent steps. The crude amylase enzyme can be precipitated and concentrated using ammonium sulphate precipitation or organic solvents. The precipitated sample can be subjected to dialysis against water or a buffer for further concentration. This can be followed by any of the chromatographic techniques like ion exchange, gel filtration and affinity chromatography for further separation and purification of the enzyme. In a method of purification of the enzyme produced by Aspergillus falvus var. columnaris, the enzyme was precipitated was followed by dialysis and then column chromatography. In a study of Amylase production by Preussia minim, the sample was precipitated using trichloroacetic acid (TCA)/acetone and applied to a Sephadex G-200 gel filtration column. The resulting fractions were pooled and further applied to a DEAE-Sepharose ion exchange column. In a study of purification and characterization of an extracellular α-Amylase from Clostridium perfringens Type A, crude enzyme concentrate was prepared by precipitation with polyethylene glycol. This concentrated sample was then separated by DEAE Sephacel chromatography resulting in three distinct amylolytic peaks. Fractions from a single peak were then collected and chromatographed on the same column, followed by separation of obtained active fractions on a Sephacryl S-100 HR column. Purification of α-Amylase produced by a mutant Bacillus subtilis strain was done in a series of steps employing precipitation with 80% ammonium sulfate, TSK Toyopeal column chromatography, ultrafiltration, dialysis and SP Sepharose column chromatography. In case of thermotolerant amylases, the cell extract obtained after centrifugation which is free of cell debris can be subjected to high temperatures, in order to denature thermolabile proteins. For purification of α-Amylase produced by Thermotoga maritima MSB8, this step was followed by anion exchange chromatography. The purity was then analysed by using SDS-PAGE. The purity can also be analysed by size exclusion chromatography wherein the molecular weight of the purified protein can be determined.

Industrial Applications of α-Amylase

α-Amylase is gaining increased attention due to its starch hydrolyzing properties and the activities that can be carried out owing to this property. There are many potential and widely used applications of this enzyme on the industrial front. Enzymes have replaced the previously used chemical methods of hydrolysis in various industrial sectors to make the process environment friendly and make processes easier.

1. Production of Fructose and Glucose by Enzymatic Conversion of Starch

Starch is used in the production of fructose and glucose syrups. This process involves three steps: Gelatinization, Liquefaction, and Saccharification. Gelatinization involves the dissolving of starch granules in water to form a viscous starch suspension. The amylase and amylopectin are dispersed into the water on dissolution. Liquefaction of starch is it’s partial hydrolysis into short chain dextrins by α-Amylase resulting in reduction of the viscosity of the starch suspension. Saccharification is the production of glucose and fructose syrup by further hydrolysis. This is carried out by glucoamylase which acts as an exo-amylase by cleaving the α-1, 4 glycosidic linkages from the non reducing terminal. The action of pullulanase along with glucoamylase yields high glucose syrup. This high glucose syrup can then be converted into high fructose syrup by isomerization catalysed by glucose isomerase. The fructose syrup obtained is used as a sweetener, especially in the beverage industry.



The hydrolysis of starch for this purpose was previously carried by acid hydrolysis followed by saccharification using enzymes. This method had many drawbacks. The acidic nature of the process required corrosion resistant material to be used for the equipment and the high temperatures would inactivate the thermolabile enzymes if the hot starch hydrolysate passes into subsequent steps. Hence, enzymatic hydrolysis is a preferred method. The α-Amylase used in the liquefaction step can be produced from various microbial sources. Thermostable amylases can be employed for hydrolysis at a high temperature. Bacillus Stearothermophilus, Bacillus amyloliquefaciens, Bacillus licheniformis and Pyrococcus furiosus are few of the many microbial sources used to produce α-Amylase that is used in starch conversion.

2. Bakery Industry

α-Amylase is added to the dough in bread baking process. This causes the starch to hydrolyze into small dextrins which can further be fermented by yeast. This increases the rate of fermentation. Also the starch hydrolysis decreases the viscosity of the dough, thus improving its texture and increasing loaf volume by rising of dough. Once the baking is done, there may be changes during storage of baked products. All undesirable changes like increase of crumb firmness, loss of crispness of the crust, decrease in moisture content of the crumb and loss of bread flavor together are called staling. The enzyme is also used as an anti staling agent to improve the shelf life and softness retention of baked goods. Though it has an anti staling effect, a slight overdose may result in gummyness of the bread. This is caused due to production of branched dextrins. In such cases pullulanase is used in combination with amylase resulting in specific hydrolysis of compounds responsible for the gummy nature of amylase treated bread.

3. Detergent Industry

The use of enzymes in detergents has increased with the changing methods of dishwashing and laundry. Consumers prefer to use cold water and mild conditions which requires the detergent to work in those imitations. Earlier the chemicals used in detergents caused harm when ingested and the conditions of dishwashing were very harsh. Hence enzymes showed the industry an alternative path. The enzymes are environmentally safe and work at mild conditions. α-Amylase is used to digest the starch containing food particles into smaller water soluble oligosaccharides. Starch can attract soil particles on to the clothes. Hence removal of starch is also important to maintain the whiteness of clothes. The stability of α-Amylase at low temperature and alkaline pH contributes to its extensive use in detergents.

4. Desizing of Textiles

Modern production processes in the textile industry can cause breaking of the warp thread. To strengthen the thread, sizing agents are used which strengthen the thread by forming a layer on it and can be removed after the fabric is woven. Starch is a preferred sizing agent as it is easily available, cheaper and can be easily removed from the fabric. The layer of starch is subjected to hydrolysis in the desizing process where α-Amylase is employed to cleave starch particles randomly into water soluble components that can be removed by washing. The enzyme acts specifically on the starch molecules alone, leaving the fibers unaffected.

5. Paper Industry

Like textiles, paper is also treated with sizing agents to protect it from mechanical strain during processing. The sizing also contributes to better quality of the paper in terms of strength, smoothness, writing and erasebility. Starch is commonly used as the sizing agent. The role of α-Amylase in the paper industry is the partial hydrolysis of starch to make it less viscous in a batch or a continuous process. This is owing to the highly viscous nature of natural starch making it unsuitable for coating on paper.

6. Fuel Alcohol Production

Among biofuels, ethanol is most widely used. As starch is an economical starting material, it is used for the production of ethanol as a biofuel. This is done in a series of steps. Firstly, the starch is subjected to liquefaction to form a viscous starch suspension. This is followed by the saccharification process where the starch is hydrolyzed by α-Amylase to yield fermentable sugars. These sugars are then fermented by yeast to produce alcohol. As an improvisation of this process, protoplast fusion between the amylolytic yeast Saccharomyces fibuligera and S. cerevisiae was performed to obtain a new yeast strain that can directly produce the biofuel from starch, eliminating the need for a saccharification step.

**Estimation- Determination of enzyme activity**

The enzyme activity is determined by measuring the reducing sugars released as a result of the action of α-Amylase on starch. Another method is to measure the extent of hydrolysis by reading the absorbance of starch-iodine complex. Few of the commonly used methods for enzyme assay are discussed below.

Dinitrosalicylic Acid Method (DNS)

In the dinitrosalicylic acid method, aliquots of the substrate stock solution are mixed with the enzyme solution. Followed by 10 min of incubation at 50C, DNS reagent is added to the test tube and the mixture is incubated in a boiling water bath for 5 min. After cooling to room temperature, the absorbance of the supernatant at 540 nm is measured. The A540 values for the substrate and enzyme blanks are subtracted from the A540 value for the analyzed sample. In a study on alkalophilic α-Amylase from Bacillus strain GM8901, the enzyme assay was done by measuring the reducing sugars by DNS method and the activity was found to be a maximum of 0.75 U ml-1 after incubation of 24 h.

Nelson – Somogyi (NS) Method

In the NS method, an aliquot of stock solution of substrate is heated at 50°C for 5min. Preheated (50°C for 5 min) enzyme solution is added to the substrate. This reaction mixture is incubated at 50°C and the reaction is carried out for 10min. After incubation Somogyi copper reagent is added to terminate the reaction. This is then incubated in boiling water bath for 40 min & cooled to room temperature. In the next step Nelson arsenomolybdate reagent is added and incubated for 10 min at room temperature. Finally water is added and the mixture is centrifuged at 13,000 rpm for 1 min and absorbance of supernatant is read at 610 nm. Haloalkaliphilic α-Amylase isolated from archaebacterium Natronococcus sp. Strain Ah-36 was subjected to NS method of enzyme assay and activity of 0.01 U/ml was recorded in the end of 35 h of incubation. The maximum activity was found to be 0.12 U/ml after 90 h and was retained even after 110 h.

Determination of Activity Using Iodine

The hydrolytic activity of α-Amylase can be determined based on the principle that starch and iodine react to form a blue colored complex. On hydrolysis of starch this complex changes to a reddish brown colored one. The absorbance can be read after the enzyme substrate reaction has been terminated. This gives a measure of the extent of hydrolysis of starch by α-Amylase.

Dextrinizing Activity

The crude enzyme sample is incubated at 92°C with soluble starch to carry out the reaction for 10 min. The reaction is terminated by cooling the reaction mixture in ice water. Iodine (KI, Iodine) is added to form a colored complex with the starch in the reaction mixture. This complex is diluted with water in order to bring the color to a measurable range that can be read at 600 nm.

Indian Pharmacopoeia Method

This method is employed to measure the enzyme activity in terms of the amount of starch (grams) digested by enzyme of a given volume. In this method various dilutions of enzyme solution in incubated with starch at 40°C for 1 h after forming the colored complex by addition of iodine, the tube that shows no blue color is used to calculate the enzyme activity.

Reduction in Viscosity of Starch Suspension

This method is used to determine the quality of flour in the baking industry. Two methods used to measure enzyme activity in terms of decreasing viscosity of starch suspension are: falling number (FN) method and Amylograph/Farinograph test. In the falling number method, the enzyme substrate preparations are assayed at 100°C. Normally malted flour has a falling number of around 400. The amylograph test employs the principle of the relationship between peak viscosity of starch slurry and the enzyme activity. The lesser the viscosity of the starch slurry, more is the activity of the enzyme. Values of 400-/600 Brabender units of the Farinograph are found optimal for flour used to bake bread.

**Organic acid- citric acid**

**Reference -** [**http://www.scielo.br/scielo.php?script=sci\_arttext&pid=S1516-89131999000300001**](http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1516-89131999000300001)

**INTRODUCTION**

Citric acid (C6H8O7, 2 - hydroxy - 1,2,3 - propane tricarboxylic acid), a natural constituent and common metabolite of plants and animals, is the most versatile and widely used organic acid in the field of food (60%) and pharmaceuticals (10%). It has got several other applications in various other fields. Currently, the global production of citric acid is estimated to be around 736000 tones/year (Química e Derivados, 1997), and the entire production is carried out by fermentation. In Brazil, almost the entire demand of citric acid is met through imports. There is constant increase (3.5-4%) each year in its consumption, showing the need of finding new alternatives for its manufacture.

**Historical developments**

Citric acid was isolated by Karls Scheels in 1874, in England, from the lemon juice imported from Italy. Italian manufacturers had monopoly for its production for almost 100 years, and it was sold at high cost. This led extensive attempts all over the world to find alternatives way for its production, which included chemical and microbial techniques. In 1923, Wehmer observed the presence of citric acid as a by-product of calcium oxalate produced by a culture of *Penicillium glaucum*. Other investigations showed the isolation of two varieties of fungi belonging to genus *Citromyces* (namely *Penicillium*). However, industrial trials did not succeed due to contamination problems and long duration of fermentation (Rohr et al., 1983). The industrial process was first open by Currie, in 1917, who found that *Aspergillus niger* had the capacity to accumulate significant amounts of citric acid in sugar based medium. He also showed that high concentrations of sugar favoured its production, which occurred under limitation of growth. In the thirties, some units were implanted in England, in Soviet Union, and in Germany for the commercial production. However, the biochemical basis was only cleared in the fifties with the discovery of the glycolytic pathway and the tricarboxylic acid cycle (TCA). Consequently, an improved process employing submerged fermentation was developed in United States (Aboud-Zeid and Ashy, 1984).

Although methods were well developed to synthesis citric acid using chemical means also, better successes were achieved using microbial fermentations, and over the period of time, this technique has become the method of ultimate choice for its commercial production, mainly due to economic advantage of biological production over chemical synthesis (Mattey, 1992). Much attention has been paid on research to improve the microbial strains, and to maintain their production capacity.

**Applications of citric acid**

Citric acid is mainly used in food industry because of its pleasant acid taste an its high solubility in water. It is worldwide accepted as "GRAS" (generally recognized as safe), approved by the Joint FAO/WHO Expert Committee on Food Additives. The pharmaceutical and cosmetic industries retain 10% of its utilization and the remainder is used for various other purposes. [Table 1](http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1516-89131999000300001#tab1) presents main applications of citric acid.



**MICRO-ORGANISMS USED FOR CITRIC ACIC 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 energy 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](http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1516-89131999000300001#tab2) 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 micro-organism are: (a) its ease of handling, (b) its ability to ferment a variety of cheap raw materials, and (c) high yields.



**Strains selection and improvement**

The two principal methods of selecting populations, namely, "the single-spore technique" and the "passage method" have been used for selecting citric acid producing micro-organisms The single-spore technique has the disadvantage that mineral acid or organic acids (gluconic acid, oxalic acid) simulate the presence of citric acid. Rohr et al. (1979) improved this method by incorporating a specific stain for citric acid (para-di-methylamino benzaldehyde), instead of using the indicator.

The most employed technique to improve citric acid producing strains has been by inducing mutations in parental strains using mutagens. Among physical mutagens, g-radiation (Bonatelli and Azevedo, 1983 ; Gunde-Cimerman, 1986 ; Islam et al., 1986) and UV-radiation (Pelechova et al., 1990) have often used. To obtain hyper-producer strains, frequently UV treatment could be combined with some chemical mutagens, e.g. aziridine, N-nitroso-N-methylurea or ethyl methane-sulfonate (Musilkova et al., 1983). By using a suitable selection technique on model medium with non-specific carbon sources, a strain yielding high amounts of citric acid from unusual substrates can be obtained from the mutants produced.

Another approach for strain improvement has been the para-sexual cycle, as first described by Pontecorvo et al. (1953). According to Das and Roy (1978), diploids displayed higher citric acid yields compared to their parent haploids, but tended to be less stable (Bonatelli and Azevedo, 1983). Protoplast fusion appeared to be a promising tool to extend the range of genetic manipulation of *A. niger* with respect to citric acid production. Kirimura et al. (1988a) studied protoplast fusion of production strains. They were able to obtain fusants with acid production capacities exceeding those of the parent strains in solid state fermentation, but not in submerged fermentation. Some other aspects of strain improvement could be the resistance to detrimental constituents of fermentation raw materials, capability of utilizing raw materials (starch, cellulose, pectin containing and other waste materials). However, there is no single effective technique to achieve hyper-producing mutants and much remains to be done in this area.

**PRODUCTION TECHNIQUES AND RAW MATERIALS**

Although citric acid is mostly produced from starch or sucrose based media using liquid fermentation, a variety of raw materials such as molasses, several starchy materials and hydrocarbons have also been employed. Rohr et al. (1983) classified raw materials used for citric acid production in to two groups: (i) with a low ash content from which the cations could be removed by standard procedures (e.g. cane or beet sugar, dextrose syrups and crystallized dextrose); (ii) raw materials with a high ash content and high amounts of other non sugar substances (e.g. cane and beet molasses, crude unfiltered starch hydro-lysates).

Several attempts have been made to produce citric acid using molasses, which is preferred due its low cost and high sugar content (40-55%). The composition of molasses depends on various factors, e.g. the kind of beet and cane, methods of cultivation of crops and fertilizers and pesticides applied during cultivation, conditions of storage and handling (e.g. transport, temperature variations), production procedures, etc. 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. Despite that, cane molasses posses difficulties in achieving good fermentation yields.

Various other agro-industrial residues such as apple pomace, cassava bagasse, coffee husk, wheat straw, pineapple waste, sugar beet cosset, kiwi fruit peel, etc. have been investigated with solid state fermentation techniques for their potential to be used as substrates for citric acid production (Pandey and Soccol, 1998, Pandey et al. 1999, Vandenberghe et al., 1999a,b, c). In fact, these residues are very well adapted to solid-state cultures due to their cellulosic and starchy nature. However, despite the fact that these solid residues provide rich nutrients to the micro-organisms, and are good substrates for growth and activity of micro-organisms, much remains to be done for developing commercially feasible process utilizing these residues (Pandey 1992, 1994, Pandey and Soccol 1998).

**Liquid fermentation**

**Submerged fermentation:**The submerged fermentation (SmF) process is the commonly employed technique for citric acid production. It is estimated that about 80% of world production is obtained by SmF. Several advantages such as higher yields and productivity and lower labour costs are the main reasons for this. Two types of fermenters, conventional stirred fermenters and tower fermenters are employed, although the latter is preferred due to the advantages it offers on price, size and operation (Rohr et al., 1983). Preferentially, fermenters are made of high-grade steel and require provision of aeration system, which can maintain a high dissolved oxygen level. Fermenters for citric acid production do not have to be built as pressure vessels since sterilization is performed by simply steaming without applying pressure. Cooling can be done by an external water film over the entire outside wall of the fermenter.

In SmF, different kinds of media are employed such as sugar and starch based media ([Table 3](http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1516-89131999000300001#tab3)). Molasses and other raw materials demand pre-treatment, addition of nutrients and sterilization. Inoculation is performed either by adding a suspension of spores, or of pre-cultivated mycelia. When spores are used, a surfactant is added in order to disperse them in the medium. For pre-cultivated mycelia, an inoculum size of 10% of fresh medium is generally required. Normally, submerged fermentation is concluded in 5 to 10 days depending on the process conditions. It can be carried out in batch, continuous or fed batch systems, although the batch mode more frequently used.



**Surface fermentation:**The first individual process for citric acid production was the liquid surface culture (LSC), which was introduced in 1919 by Société des Produits Organiques in Belgium, and in 1923 by Chas Pfizer & Co. in US. After that, other methods of fermentation, such as submerged fermentation were developed. Although this technique is more sophisticated, surface method required less effort in operation and installation and energy cost (Grewal and Kalra, 1995).

In the classical process for citric acid manu-facture, the culture solution is held in shallow trays (capacity of 50-100 L) and the fungus develops as a mycelial mat on the surface of the medium. The trays are made of high purity aluminium or special grade steel and are mounted one over another in stable racks. The fermentation chambers are provided with an effective air circulation in order to control temperature and humidity. Fermentation chambers are always in aseptic conditions, which might be conserved principally during the first two days when spores germinate. Frequent contamination are mainly caused by Penicilia, other Aspergilli, yeast and lactic bacteria (Rohr et al, 1983; Morgant, 1988). Refined or crude sucrose, cane syrup or beet molasses are generally used as sources of carbon. When applied, molasses is diluted to 15-20% and is treated with hexacyanoferrate (HFC).

**Solid-state fermentation**

Solid-state fermentation (SSF) has been termed as an alternative method to produce citric acid from agro-industrial residues (Pandey 1991, 1992, 1994, Soccol 1994, Pandey and Soccol 1998). Citric acid production by SSF (the Koji process) was first developed in Japan and is as the simplest method for its production. SSF can be carried out using several raw materials ([Table 4](http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1516-89131999000300001#tab4)). Generally, the substrate is moistened to about 70% moisture depending on the substrate absorption capacity. The initial pH is normally adjusted to 4.5-6.0 and the temperature of incubation can vary from 28 to 30°C. The most commonly organism is *A. niger.*However there also have been reports with yeasts (Maddox and Kingston, 1983; Tisnadjaja et al., 1996). One of the important advantages of SSF process is that the presence of trace elements may not affect citric acid production so harmfully as it does in SmF. Consequently, substrate pre-treatment is not required.



Different types of fermenters such as conical flasks, glass incubators and trays, etc. have been used for citric acid fermentation in SSF. Vandenberghe et al. (1999a,b) used Erlen-meyer flasks and glass columns for the production of citric acid from gelatinized cassava bagasse. Higher yields were obtained in flasks without any aeration, and very little sporulation was observed. The same yields were found in column reactors only with variable aeration. This showed great perspective to use SSF process for citric acid production in simple tray type fermenters.

**FACTORS AFFECTING CITRIC ACID PRODUCTION**

**Medium and its components**

**Carbon source:**Citric acid accumulation is strongly affected by the nature of the carbon source. The presence of easily metabolized carbohydrates has been found essential for good production of citric acid. Hossain et al. (1984) showed that sucrose was the most favourable carbon source followed by glucose, fructose and galactose. Galactose contributed to a very low growth of fungi and did not favour citric acid accumulation.Other sources of carbon such as sorbose, ethanol, cellulose, manitol, lactic, malic and a -acetoglutaric acid, allow a limited growth and low production. Starch, pentoses (xyloses and arabinoses), sorbitol and pyruvic acid slow down growth, though the production is minimal (Yokoya, 1992).

According to Kovats (1960), initial sugar concentration was critical for citric acid production and other organic acids produced by *A. niger*. Xu et al. (1989) reported that *A.niger* strains needed an initial sugar concentration of 10-14% as optimal; no citric acid was produced at sugar concentration of less than 2.5%. Honecker et al. (1989) showed that immobilized cells of *A.niger* needed lower concentrations of sucrose than free cells culture, in order to obtain high yields (200 g of citric acid/L for free cells culture, and 120 g/L for immobilized cells). Maddox et al. (1985) reported the influence of different sources of carbon on citric acid production by *A. niger* and *Saccharomycopsis lipolytica*. Glucose, maltose, galactose, xylose and arabinose were tested. Fermentation was carried out in 8 and 4 days, respectively, at 30°C and 180 rpm. Better results were found for *A. niger* with 0.45 g of citric acid/ g of glucose corresponding to 27 g/L. *S. lipolytica* produced 0.41 g/g of glucose or 9 g/L which was not so bad.

As presented previously, several raw materials can be employed successfully for citric acid production. There are some critical factors (costs, need of pretreatment), which should be considered for substrate determination. One another aspect is the presence of trace elements, which can act as inhibitors or stimulants. Consequently, sometimes it is necessary to conduce a pre-treatment, e.g.; precipitation of trace metals of molasses by potassium ferrocyanide.

**Nitrogen source:**Citric acid production is directly influenced by the nitrogen source. Physiologically, ammonium salts are preferred, e.g. urea, ammonium sulfate, ammonium chlorure, peptone, malt extract, etc. Nitrogen consumption leads to pH decrease, which is very important point in citric acid fermentation (Rohr et al., 1983, Kubicek and Rohr, 1986). However, it is necessary to maintain pH values in the first day of fermentation prior to a certain quantity biomass production. Urea has a tampon effect, which assures pH control (Raimbault, 1980). The concentration of nitrogen source required for citric acid fermentation is 0.1 to 0.4 N /liter. A high nitrogen concentration increases fungal growth and the consumption of sugars, but decreases the amount of citric acid produced (Hang et al., 1977).

**Phosphorous source:**Presence of phosphate in the medium has a great effect on the yield of citric acid. Potassium dihydrogen phosphate has been reported to be the most suitable phosphorous source. Shu and Johnson (1948) reported that phosphorous at concentration of 0.5 to 5.0 g/L was required by the fungus in a chemically defined medium for maximum production of citric acid. Phosphate is known to be essential for the growth and metabolism of *A. niger* (Shankaranand and Lonsane, 1994). Low levels of phosphate favour citric acid production, however, the presence of excess of phosphate was shown to lead to the formation of certain sugar acids, a decrease in the fixation of CO2, and the stimulation of growth. Phosphates acts at the level of enzyme activity and not at the level of gene expression (Kubicek et al., 1979). It is interesting to note that different strains require distinct nitrogen and phosphorous concentrations in the medium. In fact, nitrogen and phosphorous limitation is a crucial factor in citric acid production as there is an interaction between them. Consequently, the study of their combined effect is necessary (Pintado et al., 1993; Chen, 1994). Pintado et al. (1998) reported how the culturing modality conditions the behavior of the micro-organisms referring to the tendencies of production as a function of the levels of N and P. The author used as first order an empirical model based on rotatable design to study the effect of both nutrients. As expected, for the two studied strains, a similar behavior was noticed, showing an improvement towards low levels of N and P in submerged culture, and toward high levels in solid state culture, and with superior productions for the last one. Shankaranand and Lonsane (1994) affirmed that the specificity of solid state culture is largely due to a lower diffusion rate of nutrients and metabolites, which occurs in low water activity conditions. Consequently, strains with large requirements of N and P seems to be disfavored, due to the restriction of accessibility to the nutrients in the medium.

**Trace elements**: Trace element nutrition is probably the main factor influencing the yield of citric acid. A number of divalent metals such as zinc, manganese, iron, copper and magnesium have been found to affect citric acid production by *A. niger*. However, it is crucial to take into account the interdependence of medium constituents in SmF and, probably, in SSF. Zinc favoured the production of citric acid if added with KH2PO4. 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. Shankaranand and Lonsane (1994) noticed that there were few differences in the response of *A. niger* to metal ions and minerals in SSF and in SmF systems. SSF systems were able to overcome the adverse effects of the high concentrations of these components in the medium. As a consequence of this, the addition of chelating agents such as potassium ferrocyanide to the medium proved to be of no use.

Copper was found to complement the ability of iron at optimum level, to enhance the biosynthesis of citric acid. Manganese deficiency resulted in the repression of the anaerobic and TCA cycle enzymes with the exception of citrate synthetase. This led to overflow of citric acid as an end product of glycolysis (Kubicek and Rohr, 1978). A low level of manganese (ppm) was capable to reduce the yield of citric acid by 10%. Citric acid accumulation decreased by the addition of iron, which also had some effect on mycelial growth. Benuzzi and Segovia (1996) reported that the presence of different copper concentrations in the pellet formation medium was very important in order to enhance a suitable structure, related to cellular physiology, for citric acid production. The optimal initial CuSO4.5H2O concentration was 78 mg/L.

Magnesium is required both for growth as well as for citric acid production. Optimal concentration of magnesium sulfate was found in the range of 0.02-0.025% (Kapoor et al., 1983).

**Lower alcohols:**Addition of lower alcohols enhances citric acid production from commercial glucose and other crude carbohydrate. Appropriate alcohols are methanol, ethanol, iso-propanol 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%. The effect of methanol or ethanol have been extensively studied by many authors (Hamissa, 1978; Mannomani and Sreekantiah, 1988; Georgieva et al., 1992; Dasgupta et al., 1994).

Mannomani and Sreekantiah (1987) reported that addition of ethanol resulted in two-fold increase in citrate synthetase activity and 75% decrease in aconitase activity. Whereas the activities of other TCA cycle enzymes increased slightly. They also found that coconut oil influenced citric acid production in a sucrose medium when added at 3% (v/w). Alcohols have been shown to principally act on membrane permeability in micro-organisms by affecting phospholipid composition on the cytoplasmatic membrane (Orthofer et al., 1979). However Meixner et al. (1985) argued against a role of membrane permeability in citric acid accumulation. Ingram and Buttke (1984) found that alcohols stimulate citric acid production by affecting growth and sporulation through the action not only on the cell permeability but also the spatial organization of the membrane, or changes in lipid composition of the cell wall.

**Miscellaneous:**Some compounds which are inhibitors of metabolism such as calcium fluoride, sodium fluoride and potassium fluoride have been found to accelerate the citric acid production, while, potassium ferrocyanide has been found to decrease the yield. There are many compounds, which act in many ways to favour citric acid accumulation. Some of them are capable to impair the action of metal ions and other toxic compounds influence growth during the initial phase. Some of these are: 4-Methyl-umbelliferone, 3-hydroxi-2-naphtoic, benzoic acid, 2-naphtoic acid, iron cyanide, quaternary ammonium compounds, amine oximes, starch, EDTA, vermiculite, etc.

**Process parameters**

**pH:**The pH of a culture may change in response to microbial metabolic activities. The most obvious reason is the secretion of organic acids such as citric, acetic or lactic acids, which will cause the pH to decrease. Changes in pH kinetics depend highly also on the micro-organism. With *Aspergillus* sp., *Penicillium* sp. and *Rhizopus* sp., pH can drop very quickly until less than 3.0. For other groups of fungi such as *Trichoderma*, *Sporotrichum*, *Pleurotus* sp., pH is more stable (between 4 and 5). Besides, the nature of the substrate also influences pH kinetics (Raimbault et al., 1997).

Generally, a pH below 2.0 is required for optimum production of citric acid. A low initial pH has the advantage of checking contamination and inhibiting oxalic acid formation. A pH of 2.2 was reported to be optimum for the growth of the mould as well as for the production of citric acid (Srivastava and De, 1980) whereas, a higher pH i.e. 5.4 and 6.0-6.5 has been found optimum for citric acid production in molasses medium (Roukosu and Anenih, 1980).

**Aeration:**Aeration has been shown to have a determinant effect on citric acid fermentation (Rohr et al., 1983; Dawson et al., 1986). Increased aeration rates led to enhanced yields and reduced fermentation time (Grewal and Kalra, 1995).

The influence of dissolved oxygen concentration on citric acid formation has been examined. It is important to maintain the oxygen concentration above 25% saturation and interruptions in oxygen supply may be quite harmful (Kubicek et al., 1980). The high demand of oxygen is fulfilled by constructing appropriate aeration devices, which is also dependent on the viscosity of the fermentation broth. This is an additional reason why small compact pellets are the preferred mycelial forms of *A. niger* during fermentation (Kubicek and Rohr, 1986). When the organism turns into filamentous developments, e.g. due to metal contamination, the dissolved oxygen tension rapidly falls to less than 50% of its previous value, even if the dry weight has not increased by more than 5%. Aeration is performed during the whole fermentation with the same intensity through the medium at a rate of 0.5 to 1.5 vvm. However, because of economic reasons, it's usually preferred to start with a low aeration rate (0.1 to 0.4 vvm). High aeration rates lead to high amounts of foam, especially during the growth phase. Therefore, the addition of antifoaming agents and the construction of mechanical "defoamers" are required to tackle this problem.

**PRODUCT RECOVERY**

The recovery of citric acid from liquid fermentation is generally accomplished by three basic procedures, precipitation, extraction, and adsorption and absorption (mainly using ion exchange resins). Citric acid extraction has been described by the Food and Drug Administration (1975) of the United States and by Colin (1960,1962). Citric acid extracted by this method has been recommended suitable for use in food and drugs. Precipitation is the classical method and it is performed by the addition of calcium oxide hydrate (milk of lime) to form the slightly soluble tri-calcium citrate tetrahydrate. The precipitated tri-calcium citrate is removed by filtration and washed several times with water. It is then treated with sulphuric acid forming calcium sulphate, which is filtered off. Mother liquor containing citric acid is treated with active carbon and passed through cation and anion exchangers. Several anion-exchange resins are commercially available. 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.

**Estimation**

<https://www.jbc.org/content/156/1/33.full.pdf>

The citric acid is oxidized by manganese dioxide, in the presence of bromine, to acetonedicarboxylic acid, which is then rapidly brominated with simultaneous decarboxylation, yielding pentabromoacetone. After reduction of the excess manganese dioxide and bromine with hydrazine, the pentabromoacetone is isolated by ext.raction with petroleum ether. It is then treated with a sulfite solution, which we find destroys pentabromoacetone smoothly and rapidly, liberating all 5 bromine atoms as bromide ions. The bromide is determined by a direct argentometric titration.

<http://technologyinscience.blogspot.com/2012/08/citric-acid-production-by-aspergillus.html#.XnWujIgzbIU>

<https://chemlab.truman.edu/files/2015/07/THE-DETERMINATION-OF-CITRIC-ACID.pdf>

Citric acid is an important organic acid and it was initially being extracted from citrus fruits. Nowadays it is largely produced by microbial fermentation. Citric acid is commercially used in foods, soft drinks, pharmaceuticals, leather tanning, electroplating etc.*Aspergillus niger* is the most commonly used species for the production of citric acid. Most strains of *Aspergillus niger*which are mutants cannot oxidize citric acid and hence accumulate in culture medium. The composition of the culture medium is critical for obtaining high yield of citric acid. Its is essential to limit the growth of the fungus, so that high yield of citric acid accumulates in the medium. this can be accomplished by keeping trace metal deficiency in the medium. Acid is added to achieve low pH of 3.5. Sucrose serves as a carbon source for the production of citric acid. Ammonium nitrate is used to prevent the fermentation of oxalic acid glutamic acid.Fermentation is aerobic and can be carried out by submerged culture method.

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**Citric Acid Production Principle**
Most strains of *Aspergillus niger* which are mutants cannot oxidize citric acid and hence accumulate in culture medium. The composition of the culture medium is critical for obtaining high yield of citric acid. It is essential to limit the growth of the fungus, so that high yield of citric acid accumulates in the medium. This can be accomplished by keeping trace metal deficiency in the medium. Acid is added to achieve low pH of 3.5. Sucrose serves as a carbon source for the production of citric acid. Ammonium nitrate is used to prevent the fermentation of oxalic acid & glutamic acid. Fermentation is aerobic and can be carried out by submerged culture method.

**Citric Acid Production Medium**
Sucrose - 150 gm
Ammonium nitrate - 2.5 gm
Potassium Dihydrogen Orthophosphate - 1.0 gm
Magnesium sulphate heptahydrate - 0.25 gm
Distilled water - 1L
pH - 3.5

**Culturing Aspergilus niger**

1. Prepare the citric acid medium & dispense about 50ml in 250ml conical flask.
2. Autoclave and allow it cool.
3. Inoculate the medium with spores of Aspergillus niger & incubate it on a shaker water bath at 25oC with gentle shaking for 3-5 days.
4. After Incubation, filter the mycelium using double layered muslin cloth & measure the amount of citric acid in the filtrate by colorimetric and titrimetric methods.

**Estimation Citric Acid by Titrimetric Method**

A titration is a means of quantitative analysis in which the substance to be

measured (in a liquid solution) is reacted stoichiometrically with another reagent

(called a titrant) until it has completely reacted. The end of the reaction is usually

signaled with the appearance of a color from another non-interfering substance

called an indicator. In the case of the citric acid titration, a known amount of

orange juice is measured into an Erlenmeyer flask with an indicator solution

containing phenolphthalein (the indicator). Sodium hydroxide, at a known

concentration, is then carefully added into the sample until all of the acid has

reacted. When all of the acid has completely been neutralized, the addition of 1

additional drop of the sodium hydroxide solution, the titrant, causes the solution

to become basic. The basic solution will be marked by the appearance of a

pinkish color in the solution of orange juice.

The device used to add the titrant (NaOH) to the juice sample is called a buret. It

allows us to measure the exact amount of solution added during the titration.

Knowledge of this, the concentration of NaOH solution in moles/liter, and the

known stoichiometry of the reaction allows us to calculate the citric acid

concentration in the sample.

The filtrate obtained is titrated against an alkali of known strength using phenolphthalein as indicator. The end point is the formation of pale pink colour. The volume of alkali used for neutralization is used to find the normality and the percentage of citric acid in the sample.

1. 100ml of the filtrate is pipetted into a conical flask and 2-3 drops of phenolphthalein indicator is added to it.
2. This is titrated against 0.1N NaOH taken in the burette till a pale pink colour is formed.
3. The titration is repeated till concordant values are obtained.

**Calculation:**
**Example of Titration Results**

|  |  |  |  |
| --- | --- | --- | --- |
| No | Initial Burette Reading | Final Burette Reading | Vol of NaOH used |
| 1 | 0 | 8.0 | 8.0 |
| 2 | 0 | 8.1 | 8.1 |
| 3 | 0 | 8.1 | 8.1 |

Normality of Citric acid  =  [N(NaOH) \* V(NaOH)] / V(Citric acid)
                                      = [0.1 \* 8.1]/ 10
                                      = 0.081

% of Citric acid = [Normality \* Equivalent wt of citric acid \* 100] / Volume of filtrate
                        = [0.081\*96\*100] / 10
                        = 77.76%

N - Normality, V - Volume.
Equivalent wt of Citric acid - 96.

<http://webcache.googleusercontent.com/search?q=cache:_vRVTHGk3s0J:shodhganga.inflibnet.ac.in/bitstream/10603/72352/9/09_chapter%25204.pdf+&cd=21&hl=en&ct=clnk&gl=in>

The soluble constituent in the fruit juices may include sugar content. This will affect the specific gravity and the refractive index and the effect on the refractive index will not be the same as that onthe specific gravity. Soluble solids other than sucrose do not affect the specific gravity and the refractive index to the same extent as that of sucrose. The presence of citric acid in sugar solutions gives higher readings for specific gravity and lower readings for refractive index, compared to an equivalent amount of sucrose. The sugar content in fruit juices such as lime is very low. In practical use, the specific gravity and thus the density can be determined either by the aid of a hydrometer or by the aid of a pyknometer. The former is widely used, since it is easier, inexpensive and quicker. The latter one is more accurate but requires longer time as well as more care. The determination of the refractive index is one of the easiest and fastest methods to get information about the nutritive value of the fruit juices. The rise in the nutritive value indicates the increase in concentration. Several types of refractometers are used and the Abbe's refractometer with temperature regulation is suitable for laboratory purposes. To identify the organic acid content in the juices of citrus fruits an analysis is done with solutions of citric acid, malic acid and the clear fruit juices. For the analysis, the acoustic as well as optical parameters are used.

The freshly prepared juices of commonly available citrus fruits -lime, lime green, bitter lime and passion fruit were centrifuged at an r.p.m of 2000 for 20 minutes. To obtain clear solutions the centrifuged juices were then decanted and filtered.

Thermal opto-acoustic analysis- Thermal analysis may be defined as the measurement of physical and chemical properties of materials as a function of temperature. In practice, however, the term thermal analysis is used to cover certain specific properties only. These are enthalpy, heat capacity, mass and coefficient of thermal expansion. Thermal analysis is now extended to cover certain other physical properties, such as specific optic impedance (Zo), specific optical volume (v) specific opto-acoustic velocity (11), adiabatic compressibility (~s) and specific acoustic impedance (ZA). For the effective study of liquids, a range of temperature from 298 to 323 K have been chosen since majority of organic liquids have boiling point less than 373 K and the liquid state is intact in this range.

The internal properties of all bodies depend on the thermal motion of their molecules. When liquids are heated there occurs rearrangements in molecular configurations with respect to temperature, which results in changes of physical properties. This rearrangement is different for different liquids depending upon the type of bonds and molecular forces in them. Just as enthalpy change in a body on heating forms the basis of Differential Thermal Analysis [DTA]; change in opto acoustic property of a liquid on heating may be exploited to detect the substance present in it. As the thermal response of optical and acoustic properties is not linear, every substance has its own characteristic response depending on the molecular structure, chemical composition and chemical environment. This is the basic principle of thermal opto-acoustic analysis.
Density measurements were taken using a 12 cc double stem pyknometer. The masses of the liquids were determined using a single pan electronic balance having an accuracy of ± O.l mg. The refractive index was measured with Abbe's Research Refractometer at various temperatures with an accuracy of ± 0.01%. The ultrasonic velocities were measured by a single crystal ultrasonic interferometer at a frequency of 2 MHz having an accuracy of + 0.1 m/s.

Ethanol

<https://afdc.energy.gov/fuels/ethanol_production.html>

Ethanol is a domestically produced alternative fuel most commonly made from corn. It is also made from cellulosic feedstocks, such as crop residues and wood—though this is not as common.

Production

The production method of ethanol depends on the type of feedstock used. The process is shorter for starch or sugar-based feedstocks than with cellulosic feedstocks.

Most ethanol in the United States is produced from starch-based crops by dry- or wet-mill processing. Nearly 90% of ethanol plants are dry mills due to lower capital costs. Dry-milling is a process that grinds corn into flour and ferments it into ethanol with co-products of distillers grains and carbon dioxide. Wet-mill plants primarily produce corn sweeteners, along with ethanol and several other co-products (such as corn oil and starch). Wet mills separate starch, protein, and fiber in corn prior to processing these components into products, such as ethanol.

Making ethanol from cellulosic feedstocks—such as grass, wood, and crop residues—is a more involved process than using starch-based crops. There are two primary pathways to produce cellulosic ethanol: biochemical and thermochemical. The biochemical process involves a pretreatment to release hemicellulose sugars followed by hydrolysis to break cellulose into sugars. Sugars are fermented into ethanol and lignin is recovered and used to produce energy to power the process. The thermochemical conversion process involves adding heat and chemicals to a biomass feedstock to produce syngas, which is a mixture of carbon monoxide and hydrogen. Syngas is mixed with a catalyst and reformed into ethanol and other liquid co-products.

<https://biotechnologyforbiofuels.biomedcentral.com/articles/10.1186/1754-6834-3-16>

Bioethanol can be produced from sugar-rich, starch-rich (first generation; 1G) or lignocellulosic (second generation; 2G) raw materials. Integration of 2G ethanol with 1G could facilitate the introduction of the 2G technology. The capital cost per ton of fuel produced would be diminished and better utilization of the biomass can be achieved. It would, furthermore, decrease the energy demand of 2G ethanol production and also provide both 1G and 2G plants with heat and electricity. In the current study, steam-pretreated wheat straw (SPWS) was mixed with presaccharified wheat meal (PWM) and converted to ethanol in simultaneous saccharification and fermentation (SSF).

The use of bioethanol can reduce our dependence on fossil fuels, while at the same time decreasing net emissions of carbon dioxide, the main greenhouse gas. However, large-scale production of bioethanol is being increasingly criticized for its use of food sources as raw material. Brazil's bioethanol production consumes large quantities of sugar cane, while in the USA, corn is used. Other starch-rich grains, such as wheat and barley, are mostly used in Europe. The use of such sugar-rich feedstock causes the escalation of food prices, owing to competition on the market. Therefore, future expansion of biofuel production must be increasingly based on bioethanol from lignocellulosic materials, such as agricultural byproducts, forest residues, industrial waste streams or energy crops. These feedstocks, which are being used in second-generation (2G) bioethanol production, are abundant, and their cost is lower than that of food crops. In Europe, wheat straw has the greatest potential of all agricultural residues because of its wide availability and low cost.

To efficiently utilize lignocellulosic products, pretreatment is required to hydrolyse the hemicelluloses to make the celluloses more accessible to the enzymes. One of the most suitable kinds of pretreatment for lignocellulosic material is steam explosion. Combining steam explosion with acid catalysts is considered one of the most promising techniques for the commercialization of the process. Several studies have shown that impregnation of wheat straw with small amounts of H2SO4 before steam pretreatment results in improved sugar yields.

To obtain efficient ethanol fermentation with Saccharomyces cerevisiae, numerous nutrients, including trace metals and vitamins, are required during the process. Wheat hydrolysate, which is relatively cheap compared with chemicals, has been proven to be a potential supplement for lignocellulosic hydrolysate, because it is not only a sugar-containing material, but is also a complex nutrient source.

The production cost of ethanol is not only dependent on the yield but also on the concentration of ethanol in the fermentation broth, because of the high energy demand in the distillation step. In this step, the ethanol concentration in the broth after fermentation is increased to 94% using two stripper columns and a rectification column, which are heat-integrated by operating at different pressures. A significant increase in energy demand is observed at an ethanol concentration below 4%. A higher ethanol concentration can be achieved in the broth by adding starch-rich material to the lignocellulosic process, leading to a lower energy demand in distillation, thus reducing the production cost.



<https://www.hindawi.com/journals/tswj/2014/957102/>

Energy crisis is a growing global concern nowadays because of the dependence on petroleum-based fossil fuel which is exhausted very fast to meet the continuously increasing demands. Besides, fossil energy also has the direct impact on the atmosphere. It has been realized that fossil energy causes greenhouse gas emissions that have adverse effects on the environment. Burning of petroleum-based fuels causes the increase of CO2 level in the environment which is directly responsible for global warming. Another important concern of fossil fuel reliance is the political crisis. For example, incidence of oil supply disruption by the Middle East countries in the 1970s caused unrest in this essential sector. Consequently, it is an ongoing interest to find out a renewable and environmentally friendly source of energy for our industrial economies and consumer societies. Bioethanol in this aspect is an attractive option for renewable and sustainable energy source.

Among the advantageous properties of bioethanol as fuel energy, higher octane number (108), evaporation enthalpy, and flame speed and wider range of flammability are worth mentioning. Due to these characteristics, fuel ethanol gives higher compression ratio (CR) with shorter burning time, eventually providing a better theoretical efficiency than that of gasoline in an integrated circuit (IC) engine. Besides, it can be used as transportation fuel in various feasible ways, directly or blend with gasoline called “gasohol.” The most common blended bioethanol used in USA is E-10 containing a concentration of 10% ethanol and 90% gasoline. Brazil, on the other hand, uses pure ethanol or blended ethanol in a combination of 24% ethanol with 76% gasoline. Furthermore, a 5% of bioethanol blended with petrol can be used under the EU quality standard-EN/228 without any modification of engines, whereas, to use higher concentration of this fuel, namely, E-85 (85% ethanol), engine modification is required. Bioethanol, in another aspect, is an environmentally friendly oxygenated fuel containing 35% oxygen which is suitable to keep down the emission of particulate and nitrogen oxides as well as other greenhouse gases during combustion. Moreover, due to having lower ambient photochemical reactivity, it reduces the interference on ozone. This fuel energy is also a safer substitute to methyl tertiary-butyl ether (MTBE), a common additive used in gasoline for clean combustion.

However, for sustainability and economic viability, it is important to give concentration in cheaper ethanol production so that this fuel can compete with petroleum. Currently, industrial bioethanol production plants employ mainly two types of primary feedstocks such as starch from cereal crops and juice or molasses from sugar crops. About 60% of the global ethanol is produced from sugar crops, while the remaining 40% is produced from starchy grains. Bioethanol from lignocellulosic biomass has recently been studied extensively but still it is confined to the laboratory or pilot plant. It is easier and cheaper to use free sugar containing juice as feedstock of ethanol than starch or lignocellulosic biomass due to the nonrequirement of costly steps such as pretreatment and/or hydrolysis to get fermentable sugars.

Bioethanol can be produced directly from the free sugar containing juices of some crops, converting sucrose or monosaccharides, especially, glucose, into ethanol via fermentation with microorganisms. Sugarcane, sugar beet, sweet sorghum, and some fruits are the good sources of sugar-rich juices used as feedstocks in ethanol production. Direct fermentable juices obtained from these crops contain free sugars, especially, sucrose, glucose, and fructose, that make them more cost-effective feedstocks in fuel ethanol industry than starchy or lignocellulosic materials. Sucrose which is the major sugar in fermentable juices is readily broken down into glucose and fructose during earlier stage of fermentation by invertase enzyme, indigenously found in the periplasmic space of yeast used in the process. In a general procedure, juice is obtained from sugar crops, supplemented with ammonium sulfate or other nitrogen sources, sterilized, with pH and sugar concentration being adjusted, and then fermented using microorganisms, especially, yeast, under a suitable condition. The main disadvantages of using juice as feedstocks are the low storability and microbial decomposition.

Involvement of microorganisms in fermentation of sugars is a crucial part of bioethanol production. Some microorganisms have the ability to use glucose in the absence of oxygen for their energy, producing ethanol and carbon dioxide. This property makes them potential bioagents in fermentation technology from the beginning of its history. Sugar fermentation using single cell microorganism, that is, yeast, is one of the oldest practices in biotechnology, widely used for the production of drinking alcohol, namely, beer and wine, in the past time, while, nowadays, this practice is industrially used to produce fuel ethanol from renewable energy sources. Major characteristics of ethanologenic microorganisms to be employed in industrial plants are higher ethanol yield (>90.0% theoretical yield), tolerance to ethanol (>40.0 g/L), good ethanol productivity (>1.0 g/L/h), good growth in simple and inexpensive media, capability of growth in undiluted fermentation broth with resistance to inhibitors, and ability to retard contaminants from growth condition, for example, acidic pH or higher temperature.

Some microorganisms such as dried yeast or Saccharomyces cerevisiae, S. diastaticus, Kluyveromyces marxianus, Pichia kudriavzevii, Escherichia coli strain KO11 and Klebsiella oxytoca strain P2, and Zymomonas mobilis have been studied for ethanol production from sugar juices. Among these ethanol producing microorganisms, S. cerevisiae is the most attractive choice in fermentation due to its greater efficiency in sugar conversion to alcohol and capability of producing flocs during growth, making it easier to settle or suspend on need, and high tolerance to ethanol. Moreover, fermentation of some crop juices containing sucrose employs this yeast for its ability to hydrolyze sucrose into glucose and fructose with invertase enzyme. But the optimum temperature range of S. cerevisiae used for ethanol production is 30–35°C that leads the researchers to search for thermotolerant microorganisms. Z. mobilis, a Gram-negative bacterium, is also extensively studied over the last three decades in fuel ethanol production from grains, raw sugar, sugarcane juice, and syrup due to its ethanol tolerance and higher glucose uptake as well as good ethanol production capability. It can produce ethanol from glucose through Entner-Doudoroff pathway using the enzymes pyruvate decarboxylase and alcohol dehydrogenase. Higher ethanol yield (97.0%) and productivity of Z. mobilis were reported due to the production of less biomass and maintenance of higher rate of glucose metabolism through its ED pathway, while with S. cerevisiae ethanol yield was only 90.0–93.0%. Nevertheless, because of its narrow substrate range, this microorganism cannot immediately replace S. cerevisiae in fuel ethanol production. Culture maintenance is an essential step for effective fermentation. Microorganisms typically employed in fermentation process are heterotrophs that require a carbon and a nitrogen source to grow and survive in the culture media. Without proper media and suitable growth condition, it is difficult to get a healthy inoculum for incorporating microbial cells in fermentation broth. Based on type and strain of microorganisms, their growth condition also varies.

Bioethanol is produced mainly by three types of fermentation, such as batch, fed-batch, or continuous. In batch fermentation, feedstock is added to the fermentation vessel along with microorganism, nutrients, and other ingredients at the beginning of fermentation of whole batch followed by recovery of ethanol, while, in fed-batch mode, one or more ingredients are added to the vessel as fermentation is going on. Continuous fermentation involves a constant input of ingredients and removal of output from the fermentation vessel. The selection of most suitable mode of fermentation mainly depends on the kinetics of the microorganisms used and the nature of feedstocks. Batch fermentation is the simple fermentation process due to low cost, less control requirement, easier sterilization, and management of feedstocks as well as employment of unskilled workforce. Besides, most of the ethanol production study from juice feedstocks was carried out by batch fermentation. Fed-batch mode is broadly employed in industrial production due to compiling the benefits from both batch and continuous processes. This mode of fermentation gives some advantages over conventional batch process such as maintenance of maximum viable cell concentration, extended lifespan of cell, higher product accumulation, less inhibitory effect of higher substrate concentration, and control of several critical factors such as pH, temperature, and dissolved oxygen at a specific level through the feedback activities. Continuous fermentation that can be carried out in mainly two basic types of reactors; for example, plug flow reactor and continuous stirred tank reactor offer some advantages over batch fermentation. This mode of fermentation needs less downtime for vessel cleaning and filling giving increased productivity with lower cost.

Free cells of suitable microorganism are normally used in fermentation that carry out their metabolic function in the fermentation broth producing ethanol from sugars. However, use of immobilized microbial cells on different carriers instead of free cells in fermentation is extensively studied to improve the process which showed some technical and commercial benefits over free cell system due to changes in growth condition, physiological and morphological properties, and catalytic activity of cells. This technique enhances the productivity and ethanol yield and reduces the inhibitory effect of high substrate concentration and product. In addition, immobilization prevents cell washout in continuous fermentation that avoids separation or recycle of cells in the process. Several carriers have been reported for cell immobilization including apple pieces, k-carrageenan gel, polyacrylamide, g-alumina, chrysotile, calcium-alginate, sugarcane pieces, banana leaf sheath, and orange peel. Immobilization of S. cerevisiae can easily be carried out by enriched cells from culture media and harvested at the log phase of growth followed by entrapping into the carriers. It was reported that Z. mobilis in an immobilized cell reactor can produce increased ethanol during fermentation with the capability of tolerating high concentration of sugars.

Economic evaluation of fuel ethanol production reveals that more energy is consumed in recovery steps conducted by distillation due to low ethanol concentration in fermented broth. Therefore, increasing the ethanol content in the broth can considerably reduce energy consumption in distillation. Very high gravity (VHG) fermentation is a technique of using high concentration of sugars during fermentation with the output of increased concentration of ethanol. This is a technique employed in fermentation of the processed feedstocks containing 270 g/L or more dissolved solids, that is, free sugars. This technology exploits the enhanced and prolonged growth of microorganism in the presence of low level of oxygen and reduces water consumption, labor cost, and distillation cost with more alcohol production. However, ethanol is a toxic metabolite on yeast cells that may lead to cell lysis and death under this VHG environment with a limited ethanol concentration in the broth. Hence, viability loss of cells should be evaluated during fermentation using methylene blue stain technique or colony forming units (CFU) method.

Impact of Different Factors on Fermentation Ethanol Production

Several factors, especially, temperature, pH, fermentation time, agitation rate, initial sugar concentration, and inoculum size, have an impact on fermentation process as well as ethanol yield.

1. Temperature

Temperature is an important factor carefully regulated during fermentation as it has vital impact on the process and ethanol production. It was also reported that ethanol production depends on fermentation temperature and to some extent its concentration increases with the increase in temperature. However, high temperature is considered as a stress factor for microorganisms, which is unfavorable for their growth. They produce heat-shock proteins in response to the high temperature and inactivate their ribosomes. In addition, microbial activity and fermentation process are regulated by different enzymes which are also sensitive to high temperature since it denatures their tertiary structure eventually inactivating them. Moreover, microorganisms used in the fermentation process have optimum temperature range for their better growth. Therefore, it is necessary to predetermine an optimum temperature during fermentation for proper microbial growth as well as higher yield of ethanol. It is generally believed that the ideal fermentation temperature range is between 20 and 35°C and high temperature in almost all fermentation processes creates problem. The optimum fermentation temperature for free cells of S. cerevisiae is near 30°C, while for immobilized cells it is slightly higher probably because they can transfer heat from particle surface to inside the cells. In a study with sweet sorghum juice using immobilized yeast cells, it was reported that at 28°C ethanol yield was 75.79% followed by growing up to the maximum yield (89.89%) at 37°C. In another study with the strain S. cerevisiae BY4742 in batch fermentation, Lin et al. reported that the highest specific cell growth rate and specific productivity of ethanol were found at 30–45°C with a significant decrease in cell growth as well as in ethanol yield at 50°C. In case of Z. mobilis, the best ethanol concentration (55.57 g/L) was found at 30°C, while the lowest (4.6 g/L) was found at 40°C.

2. pH

Enhanced ethanol production through fermentation can be obtained by controlling pH of the broth as it is one of the key factors for ethanol production having direct influence on organisms as well as on their cellular processes. In general, H+ concentrations in fermentation broth can change the total charge of plasma membrane affecting the permeability of some essential nutrients into the cells. The optimum pH range for S. cerevisiae used in fermentation for ethanol production is 4.0–5.0. However, very recently, it was reported that this well-known yeast could produce ethanol from date juices even at pH 3.8, though the critical pH for this organism is 2.3. On the other hand, the highest ethanol yield was obtained using Z. mobilis adjusting the pH range of the broth as 5.0–6.0. Different optimum pH range was also reported for several feedstocks such as 2.8 to 3.4 for sugarcane juice and 4.0 to 4.5 for sucrose.

3. Fermentation Time

Shorter time in fermentation causes inadequate growth of microorganisms eventually causing inefficient fermentation. On the other hand, higher fermentation time causes toxic effect on microbial growth especially in batch mode due to the high concentration of ethanol in the fermented broth. Nadir et al. got the highest ethanol concentrations after 64 h accounting for 40.11 g/L followed by dropping to 37.24 g/L after 72 h fermentation while studying with sweet sorghum. In addition, more time is required to complete fermentation at lower temperature though ethanol yield is the lowest. For example, only 44.0% of sugar was consumed in more than 240 h producing the lowest ethanol when fermentation was carried out at 15°C.

4. Agitation Rate

Agitation plays important role in getting higher yield of ethanol during fermentation by increasing the permeability of nutrients from the fermentation broth to inside the cells and in the same way removing ethanol from the cell interior to the fermentation broth. Agitation also increases the sugar consumption and reduces the inhibition of ethanol on cells. Useful agitation rate is 150–200 rpm for yeast cells in fermentation. Liu and Shen reported the maximum ethanol yield (85.73%) at 200 rpm of agitation. Nevertheless, excess agitation rate is not suitable for smooth ethanol production due to the limited metabolic activities of cells.

5. Sugar Concentration

Initial sugar concentration is an important influencing parameter as it has the direct effect on fermentation rate and microbial cells. The actual relationship between initial sugar content and the fermentation rate is rather more complex. Generally, fermentation rate will be increased with the increase in sugar concentration up to a certain level. But excessively high sugar concentration will exceed the uptake capacity of the microbial cells leading to a steady rate of fermentation. In batch fermentation, increased ethanol productivity and yield can be obtained at higher initial sugar concentration, but it takes longer fermentation time and subsequently increases the recovery cost. Considering these facts, the optimum sugar concentration in batch fermentation was determined as 24°Bx (equivalent to 190.0 g/L). Similarly, the optimal ratio of sugar and microorganism concentration was reported as 200.0 g/L and 30.0 g/L, respectively, in an investigation with date juice fermentation.

6. Inoculum Size

Inoculum concentration does not have significant influence on final ethanol concentration but significantly affects sugar consumption rate and ethanol productivity. However, it was reported that ethanol production was increased with the increase in the initial cell numbers from 1 × 104 to 1 × 107 cells/mL and no significant difference in ethanol production was found between 107 and 108 cells/mL. Increased cell concentration within a certain range also reduces fermentation time considerably due to the rapid growth of cells in the fermentation media that immediately consumes fed sugars producing ethanol. Breisha reported that reduction in fermentation time from 72 h to 48 h was found by increasing yeast concentration from 3.0% to 6.0%.

<http://www.etipbioenergy.eu/value-chains/conversion-technologies/conventional-technologies/ethanol-fermentation>

Ethanol (C2H5OH) is a light alcohol and is a volatile, colourless, flammable liquid with a characteristic odour. It is also known as ethyl alcohol and often abbreviated as EtOH. The most common way of production is the fermentation of sugar or starch from agricultural crops by yeasts or bacteria. Ethanol can either be used as high blend (E85) in dedicated flex-fuel vehicles, or as low blend in most current vehicles without modifications. The blending rate is usually up to 10%.

PRODUCTION PROCESS

Feedstock

The feedstock for ethanol production can be any material containing appreciable amounts of sugar or substances that can be converted to sugar. Conventional production uses sugar (from sugar cane and sugar beet), starch (from corn, wheat or potatoes) or other polysaccharides. The production process of second generation ethanol, also called cellulosic alcohol, uses cellulosic feedstock (e.g. from agricultural residues) which require further pretreatment.

Metabolic conversion

In a fermentation process sugar (glucose, fructose or other monosaccharides) is converted to ethanol by microbes (mostly varieties of the yeast Saccharomyces cerevisiae), which are inoculated to the feedstock. The monosachharides originate either directly from disaccharides, which are broken up via invertase enzymes, or from starch which is hydrolysed with amylase enzymes. In addition to ethanol, water and carbon dioxide are prdouced also.

The glucose-to-ethanol reaction is represented by the equation below:

C6H12O6 + 2 ADP + 2 Pi → 2 C2H5OH + 2 CO2 + 2 H2O + 2 ATP

Common processes produce a fermentation broth with concentration of 5% - 10% ethanol per volume, as ethanol itself is toxic to the microorganisms. More advanced facilities are able to increase the concentration up to 20% due to the use of adapted and specialized yeasts.

Upgrading

The upgrading includes the recovery and concentration of ethanol from the fermentation broth. Therefore the following technological steps are applied:

Evaporation of ethanol: in this step the first evaporation of ethanol is performed in order to obtain “crude” ethanol with concentration about 45% per volume.

Rectification: in the rectification step the ethanol concentration is increased to 96% per volume

Dehydration: by dehydration the remaining azeotropic water is removed in order to obtain a required concentration of 98.7% per mass and water content below 0.3% per mass.



Schematic diagram of bio-ethanol production by fermentation process of sugar, starch and lignocelluloses feedstock

Estimation

The simultaneous saccharification and fermentation (SSF) of mixtures of cellulosic material (steam pretreated wheat straw; SPWS and presaccharified wheat meal (PWM) can be done. The liquid fractions from pretreatment, samples from acid hydrolysis and the supernatants of SSF broth were analysed by HPLC, in a chromatograph equipped with a refractive index detector. Ethanol were separated (in a Aminex HPX-87H column; Bio-Rad Laboratories) at 65°C. The eluent was 0.005 M H2SO4 at a flow rate of 0.5 mL/min.

<https://orbitbiotech.com/ethanol-estimation-by-potassium-dichromate-method/>

Most of the chemical oxidation methods are based on the complete oxidation of ethanol by dichromate in the presence of sulfuric acid with the formation of acetic acid. This reaction is highly preferred because potassium dichromate is easily available in high purity and the solution is indefinitely stable in air. The reaction that occurs between alcohol and potassium dichromate is:
2Cr2O7– + 3C2H5OH + 16H+ 4Cr+++ + 3CH3COOH + 11H2O
Dichromate (Cr2O7–, Cr(VI)) is yellowish in color and the reduced chromic product (Cr+++, Cr(III)) is intensely green. Because the absorption spectra of dichromate and chromic ions overlap significantly, Beer’s law is not obeyed. Instead, the spectra of the solution of interest must be analyzed at multiple wavelengths to calculate the individual concentrations of dichromate and chromic ions in a mixture subject to the material balance that the total number of chromium atoms must be conserved.
Proper concentration of sulfuric acid in the surrounding solution will direct the oxidation of ethanol toward acetic acid instead of acetaldehyde.

Requirements

1. Chromic acid reagent
2. Ethanol sample
3. Ethanol standard
4. Distilled water

Procedure

1. Collect 1 ml ethanol sample by distillation of the wine sample.
2. To 1ml of sample/solution, add 25 ml of chromic acid reagent.
3. Place the tubes in a water bath at 70ºC for 15 min.
4. Take out the tubes and immediately add 24 ml of distilled water to it to stop the reaction.
5. Measure the absorbance at 600 nm. Compare it with standard graph.

**Antibiotic- penicillin**

Estimation

<https://www.nature.com/articles/158910b0>

THE Kapeller-Adler1 method for the estimation of phenylalanine depends on the nitration of phenylalanine to give 3:4 dinitrophenylalanine; this is then reduced by alkaline hydroxylamine to a coloured nitroso derivative. It appeared probable that, by virtue of its phenylacetic radical, penicillin II (G) might be estimated in the same way and in the presence of penicillin I (F).