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A REVIEW ARTICLE

Recent Advances on Efficient Methods for α-Amylase Production by Solid State Fermentation (SSF)

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Abstract

Enzymes are biological catalysts which regulates biochemical reactions. They are now being used in various sectors of industry preferred than chemical catalysts because of some advantages they have: can act under-mild conditions, preventing the formation of undesirable by products, highly specific and catalyze reactions faster than chemical catalysts. Among the various industrially important enzymes, α-amylase ranks first in terms of commercial exploitation. Although amylases can be derived from several sources, production using microorganisms is economical in bulk production capacity and microbes are also easy to manipulate to obtain enzymes of desired characteristics. There are mainly two methods which are used for production of α-Amylase on a commercial scale: Submerged fermentation and Solid State fermentation. However, SSF has advantages for the production of α-amlylase over submerged fermentation (SmF), due to, superior productivity, simple technique, low capital investment, low energy requirement and less water output, better product recovery and lack of foam build-up. In addition, the use of agro-industrial waste makes solid--state fermentation (SSF) an attractive alternative method as support and carbon source for production of industrial enzymes and for various value added products(organic acids, flavor compounds, biofuels, secondary metabolites, etc).

1. INTRODUCTION

Enzymes are biological catalysts which are an indispensable component of biological reactions and regulate specific biochemical reactions (Sundarram, 2014; Tiwari, 2015)**.** The use of chemical catalysts has been used for a very long time. Chemical catalysis though widely used was very cumbersome; the disadvantages that this method poses include need for high temperature and pressure for catalysis and moderate specificity. These limitations were overcome by the use of enzymes (Sundarram, 2014). Enzymatic hydrolysis has some advantages compared to chemical methods, the biocatalysts act under mild conditions of pH and temperature, reducing energy consumption, equipments corrosion and eliminates neutralization steps. However, specificity of enzymatic catalysis can be considered as the main advantage of the enzymes use, preventing the formation of undesirable byproducts commonly observed in reactions by chemical catalysis (Ana, Maria, Heloiza, Andre, Marcelo, Gustavo, et al., 2015; Sundarram, 2014) and catalyze reactions faster than chemical catalysts (Sundarram, 2014).

Enzymes are now being used in various sectors of industry. In recent years, the potential of using several microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the

exploration of extracellular enzymatic activity (Tiwari, 2015). They are used in detergents industry; brewing industry ; paper industry, textile industry, food industry and many other industrial applications (Sundarram, 2014; Tiwari, 2015). Among the various industrially important enzymes, α-amylase ranks first in terms of commercial exploitation (Sundarram, 2014; Tiwari, 2015), accounts for 25-33% of the international enzyme market and are used in numerous industrial processes that require the partial or total hydrolysis of starch (Ana, et al., 2015; de Souza, 2010). The spectrum of applications of α-amylase has widened in many sectors such as clinical, medicinal and analytical chemistry(SINGH, 2011; Tiwari, 2015) . Besides their use in starch saccharification, they also find applications in baking, production of cakes, fruit juices, starch syrups, brewing, detergent, warp sizing of textile, paper and distilling industry, for the pretreatment of animal feed to improve the digestibility, pharmaceutical and bioconversion of solid waste etc (Anto, 2006; Couto & Sanromán, 2006; de Souza, 2010; Khan, 2011; Mobini-Dehkordi, 2012; Sharanappa, 2011; Sundarram, 2014; Tiwari, 2015). Large range of applications is the triggering factor for the industrialization of alpha-amylase production (Khan, 2011).

Enzymes belonging to amylases: endoamylases and exoamylases, are able to hydrolyse starch. These enzymes are classified according to the manner in which the glycosidic bond is attacked. α-amylases (endo-1, 4-α-D-glucan glucanohydrolase EC 3.2.1.1) are extra-cellular endo enzymes that randomly cleave the 1,4-α linkages between adjacent glucose units in the linear amylose chain and ultimately generates glucose, maltose and maltotriose units(Anto, 2006; Couto & Sanromán, 2006; de Souza, 2010; El-Fallal, Abou, El-Sayed, & Omar, 2012; IRFAN, 2012). Endo-amylolytic and debranching enzymes (α-amylases and isoamylases, respectively) reduce the degree of polymerization of the starch molecule (Amylose and amylopectin chain structure), producing linear glucose-based dextrins. These enzymes are employed in the starch liquefaction process. Exo-amylases are used in subsequent steps of the enzymatic hydrolysis of starch (Amylose and amylopectin chain structures) **(Fig-1).** These enzymes hydrolyze dextrins from the liquefaction, producing maltose (β-amylases) or glucose (amyloglucosidase, α-glucosidases and glucoamylases) syrups that are used in the food, beverage and biofuel industries (Ana, et al., 2015; Anto, 2006; El-Fallal, Abou, El-Sayed, & Omar, 2012; Hmidet, El-Hadj Ali, Haddar, Kanoun, Alya, & Nasri, 2009; IRFAN, 2012). α-amylases are one of the most popular and important form of industrial amylases and the present review focuses on production of these enzymes through Solid-state fermentation method.

2. Structural and Functional Characteristics of α-Amylase

The amylase has a three-dimensional structure capable of binding to substrate and, by the action of highly specific catalytic groups, promotes the breakage of the glycoside links(de Souza, 2010; El-Fallal, Abou, El-Sayed, & Omar, 2012). α-amylase is a classical calcium-containing enzyme (calcium metalloenzymes), which are completely unable to function in the absence of calcium (de Souza, 2010; Raul, Biswas, Mukhopadhyay, Kumar Das, & Gupta, 2014; SINGH, 2011), composed of 512 amino acids in a single oligosaccharide chain with a molecular weight of 57.6 kDa (de Souza, 2010) . The protein has 3 domains: A, B, and C as indicated in **Figure (2).** The A domain is the largest, presenting a typical barrel shaped (β/α) 8 super structure. The B domain is inserted between the A and C domains and is attached to the A domain by disulphide bond. The C domain has a β- sheet structure linked to the A domain by a simple polypeptide chain and seems to be an independent domain with unknown function. The active site (substrate-binding) of the α-amylase is situated in a long cleft located between the carboxyl end of the A and B domains. The calcium (Ca^{2+}) is situated between the A and B domains and may act in the stabilization of the three-dimensional structure and as allosteric activator (de Souza, 2010; El-Fallal, Abou, El-Sayed, & Omar, 2012; Raul, Biswas, Mukhopadhyay, Kumar Das, & Gupta, 2014; SINGH, 2011). Binding of substrate analogs suggest that Asp206, Glu230 and Asp297 participate in catalysis. The substrate-binding site contains 5 sub-sites with the catalytic site positioned at sub-site 3. Substrate can bind to the first glucose residue in subsite 1 or 2, allowing cleavage to occur between the first and second or second and third glucose residues (de Souza, 2010; El-Fallal, Abou, El-Sayed, & Omar, 2012).

3. Production of α-Amylase Enzyme

3.1 Sources of Amylases

The amylases can be derived from several sources such as plants, animals and microbes (IRFAN, 2012), but the enzymes vary in activity, specificity and requirements from species to species and even from tissue to tissue in the same organism. The amylases of microorganisms have a broad spectrum of industrial applications as they are more stable than when prepared with plant and animal α-amylases(de Souza, 2010). The major advantage of amylases production using microorganisms is in economical bulk production capacity and microbes are also easy to manipulate to obtain enzymes of desired characteristics (de Souza, 2010; El-Fallal, Abou, El-Sayed, & Omar, 2012; IRFAN, 2012; Vidyalakshmi, 2009). α-Amylase is produced by several bacteria, fungi and genetically modified

species of microbes. Bacillus species are considered to be the most important and the most widely used sources of α-amylase among the bacterial species and have been used for enzyme production using SSF (Anto, 2006). B. myloliquefaciens 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, Bacillus and stearothermophilus, Bacillus amyloliquefaciens show promising potential in a number of industrial applications in processes such as food, fermentation, textiles and paper industries. Bacillus subtilis, Bacillus licheniformis and Bacillus amyloliquefaciens are known to be good producers of thermostable α-Amylase (Sundarram, 2014; Tabassum, Khaliq, Rajoka, & Agblevor, 2014). Screening for the α-amylase producers is a key step in the production(El-Fallal, Abou, El-Sayed, & Omar, 2012) .

3.2 Alpha –Amylase 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. Currently, industrial demand of most of the enzymes is met by production using submerged fermentation (SmF), generally employing genetically modified strains(Pandey, 2000). However, the cost of enzyme production in submerged fermentation is high and it is uneconomical to use many enzymes in several processes (Anto, 2006; Pandey, 2000). The contents of synthetic media are very expensive and these contents might be replaced with more economically available agricultural by-products for the reduction of cost of the medium. This necessitates reduction in production cost by alternative methods (Anto, 2006; Pandey, 2000). Researchers are now busy in search of procedures to cut short the cost of production(Khan, 2011) and recently they have evaluated whether Solid-state fermentation (SSF) is the best system for producing enzymes. They found that SSF is appropriate for the production of enzymes, colorants and other thermo labile products or substances of interest to the food industry, especially when higher yields can be obtained than in SmF (Couto & Sanromán, 2006; de Souza, 2010). SSF holds tremendous potential for the production of enzymes. It can be of special interest in those processes where the crude fermented product may be used directly as enzyme source (Khan, 2011; Pandey, 2000; Vijayabaskar, 2012).

SSF has been defined as the fermentation process which involves solid matrix and is carried out in absence or near absence of free water; however, the substrate must possess enough moisture to support growth and metabolism of the microorganisms. The solid matrix could be either the source of nutrients or simply a support impregnated by the proper nutrients that allows the development of the microorganisms (Couto & Sanromán, 2006; El-Shishtawy, 2014; Pandey, 2003; Singhania, Patel, Soccol, & Pandey, 2009; Vijayabaskar, 2012). Solid-state fermentation (SSF) is a technology which uses a reduced reactor volume per unit of converted substrate, where fungi are applied to obtain the desired product(Grover, 2013). The low moisture content means that fermentation can only be carried out by a limited number of microorganisms, mainly yeasts and fungi, although some bacteria have also been used(Couto & Sanromán, 2006). This process is known from ancient times and different fungi have been cultivated in SSF for the production of food. Typical examples of it are the fermentation of rice by Aspergillus oryzae to initiate the koji process and Penicillium roquefortii for cheese production. China, (to produce Chinese wine, soy sauce and vinegar), Japan (commercially to produce industrial enzymes) has been used SSF extensively since ancient time. Since 1986 in Brazil a series of research projects for the value-addition of tropical agricultural products and sub-products by SSF has been developed due to the high amounts of agricultural residues generated by this country. Thus, the production of bulk chemicals and value-added fine products such as ethanol, single-cell protein (SPC), mushrooms, enzymes, organic acids, amino acids, biologically active secondary metabolites, etc. has been produced from these raw materials by means of SSF technique (Couto & Sanromán, 2006).The Main groups of microorganisms involved in SSF processes are indicated in **Table -1.**

In SSF, the cost of the substrate also plays a key role in deciding the cost of production(Khan, 2011). The use of agro-industrial waste makes solid--state fermentation (SSF) an attractive alternative method as support and carbon source for production of various value added products(Anto, 2006; Grover, 2013; Pandey, 2003). Agro industrial wastes have been reported to be a good substrate for the cost effective production of amylases and are thus attracting researchers for using agro industrial waste as a substrate for alpha amylase production(Khan, 2011). A number of such substrates have been employed for the cultivation of microorganisms to produce host of enzymes(Grover, 2013). Substrates traditionally used in SSF include rice bran, wheat bran, millet bran, barley bran, and corn and soy-bean (Vijayabaskar, 2012). Different researchers have been reported , wheat bran and rice husk, the potential of coconut oil cake using Aspergillus oryzae, a GRAS strain, the selection of a suitable low cost fermentation medium

,Glucoamylase production with an Aspergillus sp by using cheap rice flake manufacturing wastes as substrate for α-amylase production in SSF (Anto, 2006). (Anto, 2006) was evaluated , Wheat bran and two waste products obtained in processing of rice to rice flakes, coarse waste and medium waste for α-amylase production by solid-state fermentation. Among the three substrates tested highest enzyme production was observed with wheat bran ((94±2) U/g) **(see Table 2**) (Anto, 2006). Maximum enzyme production was observed after 72 h, which decreased with further incubation. Among the two rice flake manufacturing wastes tested, coarse waste gave good enzyme production. These result showed that both food and agricultural wastes are produced in huge amounts and since they are rich in carbohydrates and other nutrients, they can serve as a substrate for the production of bulk chemicals and enzymes using SSF technique(Couto & Sanromán, 2006).

3.3 Advantages of Solid-state Fermentation

The use of SSF for production of enzymes and other products has many advantages over submerged fermentation. These advantages included: the SSF process is a simple process with improved product characteristics, higher product yields, and reduced energy requirements and lower initial capital cost/investment; lower water output and easier/better product recovery, lack of foam build up and smaller reactor volume, contamination risks are significantly reduced due to the low water contents and, consequently, the volume of effluents decreases. It has been reported that it is the most appropriate process in developing countries due to the advantages it offers (El-Shishtawy, 2014; Grover, 2013; Khalaf, 2013; Sivaramakrishnan, 2007; Vijayabaskar, 2012). Another very important advantage is that, it permits the use of agricultural and agro-industrial residues as substrates which are converted into products with high commercial value like secondary metabolites. Furthermore, the utilization of these compounds helps in solving pollution problems, which otherwise cause their disposal (El-Shishtawy, 2014; Sivaramakrishnan, 2007).
Solid state fermentation (SSF) appears to possess several biotechnological advantages, such as higher fermentation productivity, higher end concentration of products, higher product stability, lower catabolic repression, mixed cultivation of various fungi and lower demand on sterility due to the low water activity used in SSF. In the SSF process, the solid substrate not only supplies the nutrients to the culture, but also serves as an anchorage for the microbial cells. The moisture content of the medium changes during fermentation as a result of evaporation and metabolic activities, thus optimum moisture level of the substrate is the most important factor for enzyme production. Solid-state fermentation has gained renewed interest and fresh attention from researchers owing to its importance in recent developments in biomass energy conservation, in solid waste treatment and in its application to produce secondary metabolites. Use of suitable low cost fermentation medium for production of alpha amylase using agricultural by-products has been also reported by (Grover, 2013).

Costs are much lower due to the efficient utilization and value-addition of wastes, have performed a detail economic analysis of the production of Penicillium restrictum lipase in both SmF and SSF. They found that for a production scale of 100 m^3 lipase concentrate per year, total capital investment needed for SmF was 78% higher than that needed for SSF. Also, SSF unitary product cost was 47% lower than the selling price. These studies pointed out that the great advantage of SSF processes is the extremely cheap raw material used as main substrate. Therefore, SSF is certainly a good way of utilizing nutrient rich solid wastes as a substrate (Couto & Sanromán, 2006). In addition, a comparison was made for cellulase production in SmF and SSF. In SmF, cellulase yields are generally about 10 g/l, and the average fermentation cost in a stirred tank bioreactor is about \$200/m^3. Thus, the production cost in the crude fermentation by SmF is about \$20/kg. In SSF, the average production level is about 10 mg/g substrate and the average fermentation cost is only about \$25/mt. Thus, the unit cost of SSF cellulase is just about \$0.2/kg(Pandey, 2000).
The production of α-amylase by submerged fermentation (SmF) and solid state fermentation (SSF) has been investigated and depend on a variety of physicochemical factors. SmF has been traditionally used for the production of industrially important enzymes because of the ease of control of different parameters such as pH, temperature, aeration and oxygen transfer and moisture(de Souza, 2010). SSF systems appear promising due to the natural potential and advantages they offer. SSF resembles the natural habitat of microorganism and is, therefore, the preferred choice for microorganisms to grow and produce useful value added products. SmF can be considered as a violation of their natural habitat, especially of fungi. Fungi and yeast were termed as suitable microorganisms for SSF according to the theoretical concept of water activity, whereas bacteria have been considered unsuitable. However, experience has shown that bacterial cultures can be well managed and manipulated for SSF processes(de Souza, 2010).

The optimization of fermentation conditions, particularly physical and chemical parameters, is important in the development of fermentation processes due to their impact on the economy and practicability of the process. The

role of various factors, including pH, temperature, metal ions, carbon and nitrogen source, surface acting agents, phosphate and agitation have been studied for α-amylase production. The properties of each α-amylase such as thermal stability, pH profile, pH stability, and Ca-independency must be matched to its application. For example, α-amylases used in starch industry must be active and stable at low pH, but at high pH values in the detergent industry. Most notable among these are the composition of the growth medium, pH of the medium, phosphate concentration, inoculum age, temperature, aeration, carbon source and nitrogen source(de Souza, 2010). Advantages and disadvantages of SSF could be summarized as **Table 3.**

3.4 Applications of solid-state fermentation

3.4.1 Production of enzymes by SSF

Enzyme production is one of the most important applications of SSF. As it is mentioned in Sec. 3.3 of this review SSF has advantages over submerged fermentation such as high volumetric productivity, low cost of equipment involved, better yield of product, lesser waste generation and lesser time consuming processes etc. The type of strain, culture conditions, nature of the substrate and availability of nutrients are the other important factors affecting yield of enzyme production. It is crucial to provide optimized water content and control the water activity for good enzyme production. Agro-industrial substrates are considered best for enzyme production in SSF. In addition to α-amylase enzyme, the following enzymes are also being produced by using solid state fermentation: Protease, Lipase, Cellulase, Pectinase, Phytase,L-glutamase, Ligninase, xylanase are to mention a few (Bhargav, 2008; Couto & Sanromán, 2006; Pandey, 2000; Singhania, Patel, Soccol, & Pandey, 2009; Tabassum, Khaliq, Rajoka, & Agblevor, 2014).

3.4.2 Production of organic acids under SSF

Fermentation plays a key role in the production of organic acids. The production of organic acids progressed with development of SSF. However, biotechnological processes for large-scale production of organic acids are still in early phases of development. Organic acids are the most common ingredients of food and beverages because of their three main properties: solubility, hygroscopic quality, and their ability to chelate. Some of the acids produced using SSF are citric acid, lactic acid, garlic acid, fumaric acid, γ-linoleic acid, and kojic acid(Bhargav, 2008; Couto & Sanromán, 2006; Pandey, 2000; Singhania, Patel, Soccol, & Pandey, 2009)

3.4.3 Secondary metabolites(Bioactive Compounds) production under SSF condition

Many practical advantages have been attributed to the production of biologically active secondary metabolites through the SSF route. Most of these are accumulated in later stages of fermentation (idio-phase). However, product formation has been found superior in solid-state processes. Problems associated with secondary metabolite production in liquid fermentation are shear forces, an increase in viscosity due to metabolite secretion, fungal morphology, and reduction in metabolite stability. Gibberellic acid is a fungal secondary metabolite produced in its stationary phase. The SSF system not only minimizes production and extraction costs, but also increases the yield of gibberellic acids (Bhargav, 2008; Pandey, 2000; Singhania, Patel, Soccol, & Pandey, 2009).

3.4.4 Flavours and Aroma production

Microorganisms play an important role in generation of natural compounds, such as fruity aroma. Flavours comprise over a quarter of the world market for food additives. Most of the flavouring compounds are produced via chemical synthesis or by extraction from natural materials. However, recent market surveys have shown that consumers prefer foodstuff that can be labeled as natural. Plants have been major sources of essential oils and flavours , but their use depends on natural factors difficult to control such as weather conditions and plant diseases. An alternative route for flavour synthesis is based on microbial biosynthesis or bioconversion. Several microorganisms, including bacteria and fungi, are currently known for their ability to synthesize different aroma compounds. Although bacteria, yeast and fungi produce aroma compounds, only a few spp. of yeast and fungi have been preferred due to their GRAS (Generally Regarded as Safe) status. Attempts to use these microorganisms in SmF resulted in low productivity of aroma compounds, which hampered their industrial application. SSF could be of high potential for this purpose. Solid-state fermentation has been used for production of aromas by cultivating Neuro-spora spp, Zygosaccharomyces rouxii, Aspergillus spp. and Trichoderma viride using pregelatinized rice, miso, cellulose fibers, and agar. Thus, the prospects of microbial production of food flavours and the recommended SSF processes for their production have reviewed (Bhargav, 2008; Couto & Sanromán, 2006; Pandey, 2000; Singhania, Patel, Soccol, & Pandey, 2009).

3.4.5 Production of bio-fuel by SSF

Ethanol is the most widely used biofuel today. Although it is easier to produce ethanol using submerged fermentation, SSF is preferred due to its lower water requirement, smaller volumes of fermentation mash, prevention of end product inhibition, and disposal of less liquid water, which decreases pollution problems (Bhargav, 2008; Pandey, 2000; Singhania, Patel, Soccol, & Pandey, 2009).

Tables and Figures

Table 1: Main groups of microorganisms involved in SSF processes: extracted from (Couto & Sanromán, 2006)

Microflora	Species	Function in SSF process
Bacteria	Bacillus species	Composting, Nattoo, Amylase
	Pseudomonas Species	Composting
	Serratia species	Composting
	Streptococcus Species	Composting
	Lactobacillus species	Ensiling, Food
	Clostridium species	Ensiling, Food
Yeast	Endomicopsis species	Tape cassava, Rice
	Saccharomyces cerevisiae	Food , Ethanol
	Schwanniomyces castelli	Ethanol , amylase
Fungi	Altemaria sp.	Composting
	Aspergillus sp.	Composting, industrial, food
	Fusarium sp	Composting, gibberellins
	Monilia sp.	Composting
	Mucor sp.	Composting, food, enzyme
	Rhizopus sp	Composting, food, enzymes,organic acids
	Phanerochaete chrysosporium	Composting, lignin degradation
	Trichoderma sp	Composting, biological control, bioinsecticide
	Beauveria sp., Metharizium sp	Biological control, bioinsecticide
	Amylomyces rouxii	Tape cassava, rice
	Aspergillus oryzae	Koji, food, citric acid
	Rhizopus oligosporus	Tempeh, soybean, amylase, lipase
	Aspergillus niger	Feed, proteins, amylase, citric acid
	Pleurotus oestreatus, sajor-caju	Mushroom
	Lentinus edode	Shii-take mushroom
	Penicilium notatum, roquefortii	Penicillin, cheese

Table 2: Production of α-amylase (U/g) by Bacillus cereus MTCC 1305 on different substrates by solid-state fermentation extracted from(Anto, 2006)

	Substrates		
Hours	Wheat bran	Course rice waste	Medium rice waste
	Enzyme production/(U/g)		
24	20±2	7±2	13±2
48	70±2	25±2	15±2
72	94±2	34±2	19±1
96	55±3	16±3	7±2

Table 3: Advantage and disadvantage of SSF over SmF

Advantages	Disadvantages

Higher Productivity	Difficulties on scale up and low mix effectively
Better oxygen circulation	Difficult to control process parameters (PH, heat, moisture, nutrient condition…)
Low –cost media	Problems with heat build up
Less effort in downstream processing	Higher impurity product, increasingrecovery product costs.
Reduced Energy and Cost requirements	
Simple technology specially for developing countries	
Scarce operational problems	
It acts as a natural habitat for several micro-organisms	

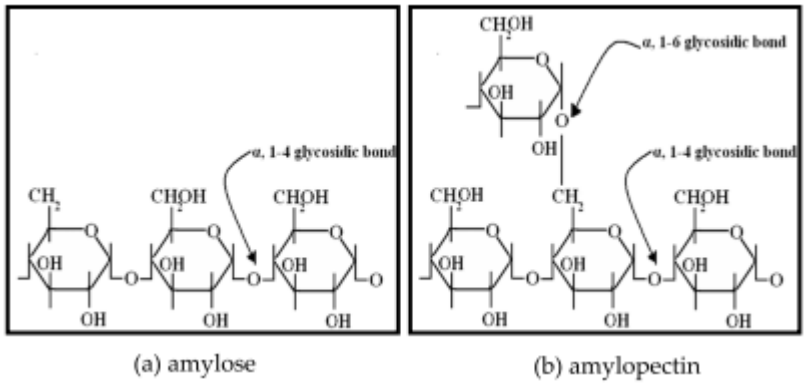

Figure 1. Amylose and amylopectin chain structure: Amylases are a class of enzymes that are capable of digesting these glycosidic linkages found in starches (El-Fallal, Abou, El-Sayed, & Omar, 2012)

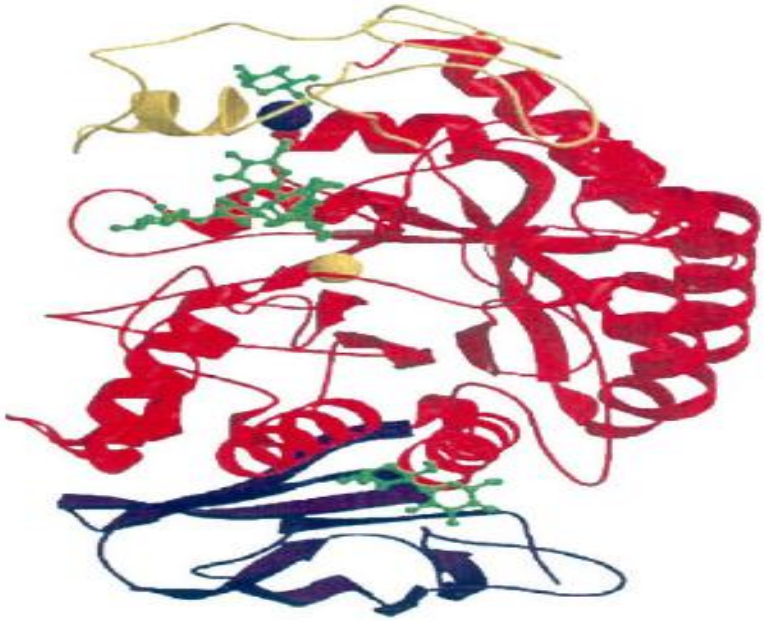

Figure 2. Structure of **α**-amylase: Domain A is shown in red, domain B in yellow & domain C in purple. In the catalytic center, the calcium ion is shown in the blue sphere and the chloride ion in the yellow sphere. The green structures are bound to the active site and to the surface binding sites(de Souza, 2010)

4 Conclusion

There have been significant developments in SSF technology over past few years. Several approaches have been applied to resolve the issues related to the biochemical engineering aspects of SSF, which include kinetics, mathematical modeling, design of bioreactors, advanced control systems to SSF processes, etc. Modeling could be a good tool for scale-up studies but such results need to be validated by experimental findings. Thus, continuous efforts would be needed to develop SSF as feasible technology for production of microbial products on commercial scale in equivalent terms of liquid fermentation technique.

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