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Role of Microbial Factory in Starch Based Industries and Different Approaches for Enhanced Production of Amylases

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Abstract. Starch hydrolyzing enzymes breaks the complex structures of starch are naturally synthesized by animals, plants and micro-organisms. These have several industrial applications like fuel production, textile industry, bread and baking, detergents, food, paper and pharmaceutical industry. The animal and poultry feed contains starch components. The digestibility of starch food in animals and birds can be increased by the addition of amylase producing micro-organisms in feed. Microbial sources gained importance over others by better quality, cost effective production and easy genetic manipulations. *Bacillus* and *Aspergillus* species are among major sources of amylases. However, enzymes market is always deficient to meet industrial demands. So, the search for new sources and improvement of enzyme stability under extreme conditions is priority. Growth optimization under different physical and chemical factors, mutagenesis and genetic modifications of microbial cells are different ways for enhanced production of amylases to meet demands of starch based industries.

Keywords: amylases, starch, *Aspergillus*, *Bacillus*, physico-chemical optimization, mutagenesis and genetic engineering

Introduction

Starch is a complex form of carbohydrates has food and non-food application. This bio-molecule is produced in various parts of plants including leaves, roots, stem and leaves. Major crops that are used industrially as a source of starch are maize, potato, wheat and tapioca. It is a reservoir of energy in chloroplast and amyloplast in the form of granules. The size and shape of granules depends upon the plant and the part of plant in which these granules are present. The starch granules are composed of amylose, amylopectin which belong to polysaccharides and also have minute quantity of protein, lipids and phosphorous (Nawaz *et al*., 2020).

Starch is a polymer of amylose and amylopection. Approximately 75% of starch consists of amylose, which is a linear form of glucose. These molecules are held together by α-1-4 linkages of glucose carbon atoms. Amylopectin that is composed of short chain of glucose molecules with α-1-4 glycosidic bonds with 5% branching pattern with α-1-6 glycosidic linkage. The complete average molecule of amylopectin contains 2000000 of glucose molecules (Maarel *et al*., 2002).

Enzymes are biological catalysts divided into six classes by International Union of Biochemistry. These lower the activation energy for converting substrate into product. Enzymes are either protein/polypeptides or glycoprotein in nature. These classes are oxidoreductases, the transferases, the hydrolases, the lyases, the isomerases, and the ligases (synthetases). Every enzyme has its enzyme commission number denoted by EC. Hydrolyses are enzymes which break C-C, C-O, C-N and other bonds by the addition of water (Karigar and Rao, 2011). There are 80 families of enzymes. The members of each family have similarity in their structure and function. The families 13, 70 and 77 consisting of enzymes, which have catalytic effect on carbohydrates basic units, which are linked through α carbon atom (MacGregor *et al*., 2001).

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Material and Method

Amylases. The first hydrolytic enzyme produced on industrial scale was an amylase. It was produced from fungi in 1894 for the treatment of digestive disorder. The amylases contribute 30% to the enzyme market in the world (Mojsov, 2012).

Alpha amylase is an extracellular enzyme (Silva *et al*., 2013), causes the hydrolysis of starch. The enzyme commission (EC) denoted number to α amylase is 3.2.1.1.alpha amylase (α-1, 4-glucan-4-glucanohydrolase) act on alpha 1, 4 glycosidic bond in starch molecules and convert this complex poly-saccharides into smaller polysaccharides units such as maltose, maltotriose and

even in simpler sugar such as glucose units. The presence of calcium ions increases the activity of alpha amylase on starch and makes it more stable (Mobini-Dehkordi and Java, 2012). Alpha amylase is produced by human, plants, animals and micro-organism but alpha amylase produced by micro-organism has greater industrial importance as compared to other sources (Abdulaal, 2018). The bacteria which are being cultivated for industrial production of alpha amylase are *Bacillus licheniformis, B. amyloliquifaciens, B. stereothermophilus, B. polymexa*, *B. subtilus*. The alpha amylase produced by *B. steriothermophilus* and *B. licheniformis* can tolerate high temperature which is required in the conversion of starch to simpler sugar on industrial scale. Among the fungal genera which are capable of producing alpha amylase are *Aspergillus, Penecillium* and *Thermomyces* (Souza and Magalhaes, 2010).

Alpha amylase is used as liquefying or saccharifying effect. If the alpha amylase produces free sugar from starch, it is saccharifing if it decreases the viscosity of starch and no free sugar is produced and it is called liquefying. The saccharifying alpha amylase may produce maltose, maltotetrose, maltopentose or maltohexose. The activity of alpha amylases produced by most of the organisms is high at neutral pH (Vaidya *et al*., 2015).

It is the member of amylase group that is least evaluated. Its EC number is 3.2.1.2. It is an exoenzyme. It is called 1, 4 α glucan maltohydrolase and target α 1, 4 glycosidic bonds present in terminal glucose molecules and produces maltose. The beta amylase of different sources is different from one another moreover beta amylase is not present in animal tissues (Das *et al*., 2011). Beta amylase from plants and animals has 30% homology (Filiz and Koc, 2014).

It is exo-enzyme of amylase. It is mainly produced by fungi. However, animal plants and other micro-organisms are also capable for the production of this enzyme. Its EC number is 3.2.1.3 and breaks both α-1, 4 and α-1, 6 glycoside bonds present in starch and yields glucose molecules (Maarel *et al*., 2002). Gluco-amylase is more stable because of the presence of extensive glycosylation. It changes the anomeric form of glucose from alpha-d-glucose to beta-d-glucose, while releasing it from starch during hydrolysis process. So, it is a sachharifying enzyme. It is also called γ amylase (aminoglycosidase). It is the most effective at acidic pH (Banerjee and Ghosh, 2017).

De-branching enzymes cause the hydrolysis of α 1-6 glycoside linkages present in amylopectin molecules of starch. So, these help in the metabolism of starch (Wei *et al*., 2012). Pullalunase and isoamylase are examples of de-branching enzymes (Siew *et al*., 2012). These cause the transformation of glycosidic bond from one molecule (donor) to another molecule (acceptor). These enzymes break the, 1-4 glycosidic bond. These also help in the formation of new glycosidic bond as well as this group includes amylomaltase and cyclodextrin glycosyl tranferases (Maarel *et al*., 2002).

Sources of amylases. Amylase can be obtained from various sources including plants, animals and micro-organisms. As per industrial level 50% enzymes are obtained from fungi, 35% by bacteria and 15% from animals and plants (Deckers *et al*., 2020).

Alpha amylase is found in milk of human (Jones *et al*., 1982). Salivary glands also produce α-amylase to start the digestion of starch in oral cavity and convert it into glucose and maltose (Nater and Rohleder, 2009). Alpha amylase is isolates from the muscle and intestine of pig parasite *Ascaris suum* (Zoltowska, 2001). Plants also produce α-amylase for hydrolysis of starch in seed that are in germination stage or in other tissues (Huang *et al*., 1992) such as cassava plant (Tangphatsornruang *et al*., 2005).

Among micro-organisms *Bacillus* spp. are famous for α-amylase production. The most important are *B. liquefaciens, B. licheniformis* and other *Bacillus* spp. However, α-amylase production has been detected in S*treptomycin albus* (Rameshkumar and Sivasudha, 2011). *B. subtilis* and *B. stearothermophilus* are also known for α-amylase production. However, fungal amylase is preferred over amylase from other sources as fungi have more acceptable GRAS status (Gupta *et al*., 2003). Filamentous fungi are well known for extracellular enzyme production. *Aspergillus* spp. are mostly used for α-amylase production by solid state fermentation (Sivaramakrishnan *et al*., 2006). *A. niger*is most important specie that is capable of producing α-amylase so far. *A. flavus* is also α-amylase producer (Monga *et al*., 2011). Other fungal species include *A. oryzae* (Abdullah *et al*., 2011), *Penicillium janthinellum* (Sindhu *et al*., 2009). *Saccharomyces cerevisiae* is a unicellular fungi also a amylase producer (Haq *et al*., 2002) another, α-amylase producing strain is *Pycnoporus sanguineus* (Pandey *et al*., 2000). Extremophiles are also producer of amylase. Amylase from *B. licheniformis* isthermophilic, while source of psychrophilic amylase

is Alteromonas haloplanktis (Bhatt *et al*., 2020). Beta -amylase is mainly present in plants. In plants it produces maltose and gives sweat taste to fruits. It is absent in animal tissues. Micro-organisms have also been reported for beta-amylase production. Bacterial species that have the potential of producing β-amylase are *Bacillus, Pseudomonas*, *Clostridium* and some *Actinomycetes* such as *Streptomyces*. *Rhizopus species* are the fungal candidates for the production of β-amylase (Olufunke and Azeez, 2013).

Unicellular (Yeast) and multicellular fungus produces glucoamylase. *Aspergillus* is famous for its production. *Rhizopus* is also able to produce glucoamylase (Far *et al*., 2020). De-branching amylases are produced by *Bacillus species, Klebsiella species* and *Geobacillus species* (Siew *et al*., 2012). Food waste is a global issue. It is the mixture of wastes from houses, industries. Food waste consists of 30% to 60% of starch material 5 to 10% protein and 10 to 40% lipids. So, a cocktail consisting of hydrolases can be used to release nutrients from food waste. Amylases are one of these enzymes (Msarah *et al*., 2020).

Micro-algae are being preferred over other sources of biomass because of its rapid growth rate. Micro-algae can be used for the production of biofuels. Bio-ethanol is produced from the bio-mass of micro-algae. This bio-ethanol is mixed with gasoline and used in vehicles in order to replace the petrol. The micro-algae which are being used are *Spyrogira, Dunaliella* and *Chlorella*. The industrial yeast *saccharomyces cerevicae* is being used for bio-ethanol production from micro-algae. This species does not possess the ability of amylase and cellulose production. So, in a pre-treatment the biomass of micro-algae is treated with *Aspergillus niger* which cause the hydrolysis of polysaccharides into simpler sugar. These sugars are further utilized by yeast to produce bio-ethanol (Singh and Trivedi, 2013).

Amylases from plants and animal are active under normal physiological parameter. However, due to divert in existence of micro-organisms, microbial enzymes are stable under harsh conditions too. Archae bacteria exist in extreme conditions of temperature, pH and salt concentration so produce extremozymes. Extremozymes are stable and remain active under extreme conditions typical of many industries (Dumorné *et al*., 2017).

Results and Discussion

Applications. Amylases have an important role in starch based industries. This hydrolyses the insoluble starch as well as aqueous suspension of starch granules (Presecki *et al*., 2013). These are used in starch liquefaction, paper industry, textile industry, food industry, pharmaceutical industry, fermentation, brewing and detergents and baking (Silva *et al*., 2013).

Alpha amylase is produced in GIT tract of birds in very low amount (Aderibigbe *et al*., 2020). In order to improve the digestibility of starch based diets in birds α amylase can be used as feed supplements (Onderci *et al*., 2006). It is also used in fruit juices, starch syrups, in making cakes and in distilling industry (Kiran and Chandra, 2008). In bread making fungal α-amylase is added in dough preparation. This amylase converts the starch into dextrin that is further fermented by yeast into carbon dioxide and alcohol. This addition of α-amylase increases the volume of loaf and improves its texture. In bread making stalling causes economic loss to baking industry. Stalling is the happening of undesirable changes in bread during storage. Many additives are added in order to reduce stalling in order to increase shelf life of bread. One of these additives is α-amylase (Maarel *et al*., 2002).

Different carbohydrates are used as sweetening agent. Fructose is sweeter in taste than the glucose. So, glucose is converted to fructose either chemically or by the use of enzyme. However enzymatic method is better for this purpose α-amylase is used. Corn syrups with high fructose contents are used in different baked goods and in soft drinks are prepared by α-amylase in order to convert glucose into fructose (Kumar *et al*., 2012). In textile industry it is used for de-sizing purpose (Kuddus *et al*., 2012). Starch is used as sizing on yarn before weaving in order to give strength during weaving after that dyes are applied to cloth. Starch paste on cloth resist the dying process. So, starch is removed by α amylase. It is called de-sizing (Mojsov, 2012).

It is used to remove the haze formation in fruit juices and saves time in beer making by speeding the mashing process in beer making (Kuddus *et al*., 2012). In detergents, α-amylase increases the efficiency of detergent by removing stains of starchy food such as gravies and chocolates. It is used for wastewater treatment coming from food processing plants (Mojsov, 2012). Coating of paper with viscous starch paste disturbs the quality of paper. So, α-amylase is used to make the starch paste less viscous in order to coat on paper (Kuddus *et al*., 2012). Glucoamylases is used to make glucoe either in solution or in crystalline form.

It is also used in dough making and used as anti-stalling agent and alcohol preparation (Kumar *et al*., 2012). Beta amylases along with pullalunases are used in sacharification process in order to yield sugars. The alpha 1,6 glycosidic bonds causes resistance in action of starch hydrolysing enzymes. So, de-branching enzymes such as pullalunases are used. These are used to prepare maltose and fructose syrups. These are also used for the synthesis of starch which contains higher amount of amylase. Boards and papers are prepared from starch containing higher amount of starch. De-branching enzymes are being utilized in preparation of detergents along with alpha amylases and in beer making. It is reported that these are also being used in medical in order to control plaques on teeths (Siew *et al*., 2012).

The amylase producing fungal strains are being used for bio-ethanol production from micro-algae in order to overcome the problems of fuels in today's world (Sing and Trivedi, 2013). Enzymes are being used in poultry feed. The utilization of enzymes as poultry feed has several advantages. Enzymes increases the digestibility of nutrients, increases the intake of feed, causes increase in body weight and decreases the habit of picking of vent etc. Among other enzymes, amylases are also added to poultry feed. The amylase is part of poultry feed in young chicks (Khattak *et al*., 2006). In case of broiler, after hatching the organs of GIT are under the process of development, so amylase production is very less from 4 day upto 21 day. This retarded the growth of broiler. So, there is need to supply amylases from external sources (Gracia *et al*., 2003).

Submerged vs Solid state fermentation. Amylases from microbial sources are obtained by the process of fermentation. Both solid state and submerged fermentation can be used for amylase production. The handling in submerged process is easy and secondly environmental factors such as temperature and pH for enzyme production can be controlled easily. The medium used for submerged fermentation is synthetic medium used with starch supplementation for bacterial amylase production. The use of synthetic medium is little bit costly. In solid state fermentation agricultural wastes are used that decreases the cost of production of amylase (Mrudula and Kokila, 2010). In case of solid state fermentation agricultural waste material with moisture is used as substrate. These agricultural waste materials include wheat, rice and soy etc. Solid state is usually performed for fungal fermentation for α-amylase production. But solid state fermentation can be used for amylase production from bacteria as well. Different factors influence the growth of micro-organism. The micro-organism growth of is related to amylase production. These factors include temperature, pH, moisture content, carbon source, nitrogen source, metal ions and surfactants (Kumari *et al*., 2019; Sivaramakrishnan *et al*., 2006).

Physico-chemical variations for enhanced amylase production. Just like all the enzymes, amylases are specific to their substrates. The substrate for amylases is mainly starch. However, the other substrate could be maltose, amylose, amylopectin and dextrin. The enzyme substrate specificity keeps on changing with respect to organism whether it is alpha amylase producer or beta amylase producer. However, all these act mainly on starch. The stability and activity of amylases is pH dependent. The activity and stability of amylases which are being produced by different micro-organisms is in the range of 2 to 12. Just like pH the amylases also dependent on temperature. Keeping in view successful research was conducted to find amylases which are able to tolerate the extreme values of temperatures and other than physical factors some metal ions help in the stability of amylases. Calcium is one of those which promote the stability of alpha amylase. Certain metals ions inhibit the activity of amylases, such as heavy metals, bovine serum albumen, EDTA, iodoacetate etc. (Saranraj and Stella, 2013).

Different factors have influence production of amylases including substrate (carbon sources) such as wheat, rice, temperature, pH, inoculum size and volume, incubation period and temperature and different supplementary sources such as nitrogen in the form of nitrate salts; peptone and yeast extract (Lalitha *et al*., 2012).

Different researchers conducted the experiments in order to check the impact of physical and chemical factors in order to get the maximum yield of enzyme. Shafique *et al*. (2009) studied the effect of pH. The resulted indicated maximum enzyme activity at 4.5 pH such as Erdal and Taskin (2010) studied temperature, pH supplements and moisture contents for fungal growth in order to get high amount of enzyme. The fungus *Penecillium expansum* was isolated from Loquat kernels. Loquat kernels flour was used as a source of carbon and supplemented with peptones. In this study pH 6, temperature 30 °C, particle size of substrate 1 mm and humidity 70% for 6 days of incubation period proved to be optimum condition for maximum enzyme

production. Activity of alpha amylase under these optimum conditions was 1012 U/g of flour of Loquat kernels.

The effects of different types of medium observed by Khokhar *et al*. (2011). In this study *Penecillium* species, *Aspergillus* species and *Trichoderma* species were used for screening their amylose production ability. The growth and amlolytic ability of fungi depends on medium. For example *Penecillium granulatum*, *Aspergillus reprai* and *Aspergillus spenuleus* growth was good on starch agar, while another species *Aspergillus nidulans* showed retarded growth on starch agar. So, a suitable substrate has an important role for fungal growth and enzyme production.

The enzyme production examined by (Balkan *et al*., 2011) providing metal ions along with other physical and chemical conditions. Different substrates were used for solid state fermentation using *Trichothecium roseum* at pH 7, under 85% humid conditions for a period of 8 days with 1000 um size of substrate particles at 30 °C. The substrates were wheat bran, rice husk and oil of sunflower meal, leaves of corncob with supplementation of metal ions, urea and lactose as inducer for amylases production. The maximum amylase (1048 U/g) was produced under these conditions using wheat bran as substrate.

It is concluded (Irfan *et al*., 2012) that with other requirements detergents also effects on enzyme production. The conditions were optimized for *Aspergillus niger* and *Rhizopus oligoporous*. The optimum conditions for *Aspergillus niger* in solid state fermentation using wheat bran as a source of starch include temperature 30 °C, 5% inoculums size, pH 5 and incubation period consists of 96 h for *Rhizopus oligosporous* pH 6, temperature 35 °C and inoculums size 10% are optimum conditions for maximum production of amylase. However, in both cases supplementation of medium with maltose, tween 80, yeast extract and nitrate and sodium salts increases the amylase production. In case of *Aspergillus niger* it increased from 2.3 to 4.4 IU, while in *Rhizopus olgoporous* it increased upto 3.2 IU.

The percentage of substrate showed by Doss and Anand (2013) influence the enzyme yield as well. This study suggested the optimal starch concentration (2%), incubation temperature of 60 °C and pH 6 for *Penecillium chrysogenum* to produces increased amount of alpha amylase.

Mutagenesis. Other approach for enhanced production of amylase is the sekection of the best mutant variants. Mutants are either produced by mutagens (physical like radiations and chemicals) or by genetic approaches not only help in efficient amylase production but also the increased stability in extreme condition of physical parameters. All factors studied by Rajasekar and Dhamodharan (2013) that affect the micro-organism growth for enzyme production along with incubation period. The enzyme production ability of micro-organisms can be increased by mutagenesis (exposure to UV light) and by genetic recombination methods (Das *et al*., 2011). The production of amylases is an inducible operon model. Several sugars act as an inducer in order to activate the amylases production gene. These inducers include maltose, isomaltose in Aspergilla. Different methods have been used to enhance the production of amylases. Mutagens are one of these. UV rays have been used to produce mutant strains on different microbial species including fungi such as *Rhizopus* and *Aspergillus* (Ruaida *et al*., 2021). In a study of (Singh *et al*., 2016), UV light and chemical mutagens were used (ethidium bromide) to mutate *Aspergillus fumigatus*. This revealed an increase in amylase activity by a value of 44.52% as compared to wild type *Aspergillus fumigatus*. After exposure of ethedium bromide the amylolytic activity of *Aspergillus fumigatus* increased to 50.03% (Singh *et al*., 2016).

Recombinant technology for enhanced and extremozymes. The advanced approach for enhanced enzyme approach is genetic manipulation. Genetically modified organisms having amylase gene with higher copy number are developed and expressed proteins are purified for downstream application. Genetic engineering helps to selected best producer among genetic variants (Subash *et al*., 2017).

Conclusion

Amylases from microbial factories are good source to meet industrial demands. Conventional and advanced approaches enhance the production of amylases to meet the demands of starch based industries.

Conflict of Interest. The authors declare that they have no conflict of interest.

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