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Recombinant amylase and its production: A review

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ABSTRACT

Amylases are a type of hydrolytic enzyme that is present in a diverse range of organisms across the microbial, plant, and animal domains. The hydrolysis of starch and related polymers yields specific sugar products in the presence of amylolytic enzymes. Amylolytic enzymes are widely utilized in industry for the manufacturing of syrups that comprise glucose, fructose, and maltose. The advancement of molecular techniques such as recombinant DNA technology (RDT) and genetic engineering has increased the production of enzymes in the manufacturing industry. The present review discussed the modern methodology of recombinant DNA technology, which involves identifying a suitable amylase gene, incorporating it into an appropriate vector system, transforming a competent bacterial host to generate a substantial volume of recombinant protein, and subsequently purifying the protein. Amylase manufacturing applies submerged fermentation (SmF) and solid-state fermentation (SSF) techniques that are frequently employed in bioprocessing. It is well known that the genome of an organism plays a significant role in influencing its productivity, despite the fact that optimizing cultural conditions may increase yield. The increased utilization of amylases across various domains in recent decades has resulted in the need to enhance both the quantity and quality of enzymatic yields. This has been achieved through strain enhancement, medium optimization, and exploring more effective fermentation processes. These enhanced strains possess distinctive advantageous characteristics and hold promise for decreasing operational expenses. Protein engineering has been extensively explored by researchers through the use of protoplast fusion and transformation techniques for mutagenesis and genetic recombination. The aim is to develop strains that exhibit enhanced enzyme productivity or other advantageous characteristics. We summarized some of the effective approaches for enhanced production of amylase in microorganisms.

Keywords- Amylase, Starch, SSF, SmF, RDT

INTRODUCTION

Amylases have been known to break down starch into smaller sugar molecules through hydrolysis. The earliestenzyme for the breakdown of starch was identified in wheat by Kirchhoff in 1811, leading to subsequent advancements in amylase research. In 1925, Kuhn designated the nomenclature of α-amylases based on the alpha configuration of hydrolysis products (Peyrot et al., 2016; Tiwari et al., 2015; Maw et al., 1991). However, Industrial amylase enzyme production from a fungus was started in late 1894 and was largelyemployed for intestinalillnesses. In 1917, Boidin and Effrontesta blushed the industrial production of α-amylase through bacterial cultures usingliquid-state fermentation (LSF) techniques (Hardin et al., 2011). Afterward, theutilization of LSF for enzyme production has been widely adopted worldwide. Prior to advancements, fungal amylase was widely manufactured in the US using solid-state fermentation (SSF) methodologies, which were first introduced by Takamine in 1914. Under kofler and coworkersfurther investigated the potential of SSF for bacterial α-amylase production, and the proficiency in SSF techniques at CFTRI in India, along with cost-effective benefits, prompted a renewed interest in the SSF process for α-amylase production in late 1985 (Singh et al., 2011;Lonsane et al., 1990). Microorganisms are widely used to produce amylase enzymes due to their technical benefits and cost-effectiveness, surpassing the chemical hydrolysis method. Amylases are present in various kingdoms including microbial, plant, and animal, and can break down starch and related polymers, producing distinct products(Imran et al., 2016; Avwioroko et al., 2018). The food industry extensively utilizes amylolytic enzymes, including to produce glucose, high fructose corn, and maltose syrups, reducing viscosity, and minimizing haze formation in juices and brewing, baking, paper, textile, and detergent industries. The applications of amylolytic enzymes have also extended to clinical and analytical chemistry (Raveendran et al., 2018; McKelvey et

al., 2017; Simpson et al., 2012). However, substrate and product accumulation and enzyme instability can hinder the enzymatic process. Current research focuses on developing novel amylases with different factors such as elevated pH and various temperature stability, increased catalytic rate, improved starch gelatinization, reduced media viscosity, and decreased susceptibility to microbial contamination, to expand their application domains.

Recombinant amylase

Recombinant DNA technology and genetic engineering are modern molecular methodologies that have been utilized to enhance enzyme production, particularly the production of amylase(Khan et al., 2016; Glick et al., 2022). The process of producing amylase through recombinant DNA technology involves several distinct stages, including identifying a suitable amylase gene, inserting the gene into an appropriate vector system, transforming it within a proficient bacterial host to generate a substantial quantity of recombinant protein, and subsequently purifying the protein for downstream applications. In recent years, there have been advancements in the expression of α-amylase from different sources using recombinant DNA technology (Gopinathet al., 2016). For instance, Wang and colleagues (2016) reported on the production of an extracellular thermostable enzyme in *Pyrococcusfuriosus*, although in low quantities. However, the expression of the enzyme in *Bacillus amyloliquefaciens* resulted in a significant increase in its production and demonstrated favorable stability at elevated temperatures and lower pH levels, resulting in a novel amylase with significantly higher yields compared to those produced in other microorganisms (Wang et al., 2016). Similarly, the cloning and overexpression of α-amylase encoding genes from other sources have been achieved in various microorganisms, leading to enhanced enzymatic production processes. For example, Li and colleagues (2015) identified and cloned the gene for α-amylase AmyM from *Corallococcus* sp. strain EGB in *E. coli*, achieving overexpression and purification of the enzyme using Ni-NTA affinity chromatography. Afzal-Javan and colleagues (2013) successfully cloned and transformed an α-amylase derived from *B. subtilis* into *Saccharomyces cerevisiae*, while Karakas and colleagues (2010) achieved the cloning and overexpression of α-amylase from *B. subtilis* PY22 via recombination in *Pichia pastoris*.

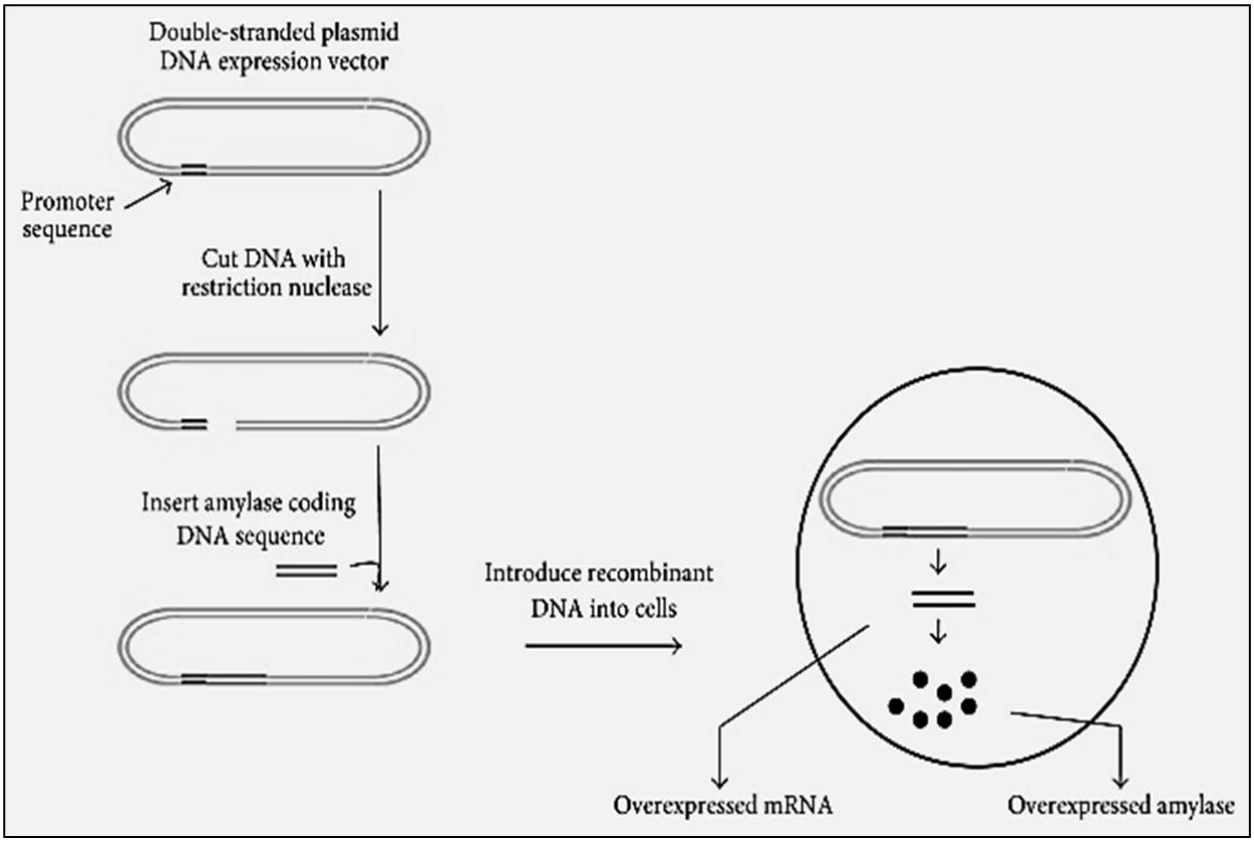

Fig.1: The utilization of recombinant DNA technology in the production of amylase. The methodology involves the identification of an effective amylase gene, its integration into an appropriate vector system, subsequent transformation into a proficient bacterial system to produce a heightened quantity of recombinant mRNA, and ultimately, the overexpression of amylase from the bacterial system (adapted from Gopinath et al., 2017).

Directed evolution has become an essential technique in genetic manipulation experiments, enabling the modification of desired properties in enzymes. The utilization of this method has been employed to augment the pH stability and specific activity of α-amylase that originates from *B. amyloliquefaciens.* According to Demirkan et al. (2005), the mutant enzyme demonstrated a fivefold enhancement in activity in comparison to the wild-type enzyme via directed evolution. The attainment of thermostability in a maltogenic amylase sourced from *Thermus sp.* strainIM6501 was accomplished through the utilization of DNA shuffling via random mutagenesis. According to Kim et al. (2003), the amylolytic activity of the mutant strain exhibited a rise in the optimal temperature range from 50 to 75°C. The acid stability of α-amylase that originates from B. licheniformis was improved by means of site-directed mutagenesis, as evidenced by another study. The mutant enzyme displayed improved acid stability as compared to the wild-type enzyme, making it suitable for use in acidic conditions (Liu et al., 2017).Recent developments in directed evolution techniques have helped to acquire specific properties for α-amylase enzymes, including great pH stability, high-temperature stability, and a broad range of acid stability. These modified enzymes can find applications in various industries, including food and beverage, pharmaceutical, and chemical industries (Pouyan et al., 2022).These studies demonstrate the potential of recombinant DNA technology and genetic engineering to enhance enzyme production and pave the way for the development of new and improved enzymes for a wide range of applications.

Production of amylase

Amylase is an enzyme that catalyzes the hydrolysis of a certain substrate like starch, ultimately forming one or more products. The lock and key model describe the mechanism by which enzymes interact with their substrates (ROBYT et al., 1984; Butterworth et al., 2011). In the field of amylase production, two primary methods are commonly employed: submerged fermentation (SmF) and solid-state fermentation (SSF). The investigation of α-amylase production through both methods has been conducted, with the outcome contingent upon various physicochemical factors. SmF has conventionally been employed for the generation of enzymes that hold significant industrial value due to the convenient regulation of various factors, including pH, temperature, aeration, oxygen transfer, and moisture (Subramaniyam et al., 2012; Panday et al., 2008). Many studies have investigated the impact of several factors, such as pH, temperature, metal ions, carbon and nitrogen sources, surface-acting agents, phosphate, and agitation, on the production of α-amylase. It is essential to ensure that the characteristics of every α-amylase, including thermostability, pH profile, pH stability, and Ca^{2+} independence, align appropriately with its intended use. SSF appears as the preferred choice for microorganisms to grow and produce value-added products promising because of its different advantages and resembling the natural habitat of microorganisms (Souza et al., 2010; Singhania et al., 2009). The optimization of fermentation conditions, specifically the physical and chemical parameters, holds significant importance in the advancement of fermentation processes owing to their influence on the feasibility and economic viability of the process. Solid-state fermentation is a valuable technique for numerous microorganisms that require a reduced moisture content to facilitate their growth. The materials utilized as substrates in this methodology include paper pulp, bagasse, and bran (Manan et al., 2017). SSF offers several advantages over SmF, including cost-effectiveness, the ability to utilize substrate slowly and for a longer period of time, high volumetric productivity, high product yield, and reduced effluent generation (Pandey et al., 2008b; Pandey et al., 2000). Due to modern techniques or inventions, the processes of solid-state fermentation can be easily done by using a bioreactor (Suryanarayan et al., 2001). The parameters that are particularly important include the formulation of the growth medium, the pH level of the medium, the concentration of phosphate, the age of the inoculum, the temperature, the aeration, as well as the sources of carbon and nitrogen. The utilisation of SmF has been reported as a method for the retrieval of secondary metabolites, as stated by Couto et al. (2006). SmF involves the utilisation of liquid substrates, such as broths and molasses, and the direct secretion of the product into the fermentation broth. A continuous supply of substrate is necessary due to its rapid utilisation. This methodology is appropriate for the proliferation of microorganisms, such as bacteria, which require elevated levels of moisture for their development. SmF offers several benefits, including the ability to utilize modified organisms to a greater extent compared to SSF, ease of purification and sterilisation, and convenient control over process parameters such as pH, moisture, oxygen level, and temperature. SmF also exhibits certain drawbacks, including limited product yield, significant water disposal requirements during downstream processing, and high costs (Sundarram et al., 2014; Kumar et al., 2012). In conclusion, the selection of fermentation method is dependent on the type of microorganism and product desired. The physicochemical factors in the fermentation process should be optimised to ensure optimal product yield and economic viability. Both SmF and SSF have advantages and disadvantages, and the appropriate method should be selected based on the specific circumstances.

Enhanced amylase production with improved strains

Microorganisms have an innate control system that, in most cases, leads to the synthesis of commercially significant metabolites in quantities that are significantly lower than what would be optimal. According to Parekh et al. (2000), even though optimizing the cultural conditions could potentially increase the yield, the genome of the organism has a significant role in determining the level of productivity. The exponential increase in the utilization of amylases across

various fields in recent decades has led to the need for both quantitative enhancement and qualitative improvement through the improvement of strains, the optimization of the medium, and the search for efficient fermentation processes to achieve higher enzymatic yields (Bisht et al., 2012). These upgraded strains may not only display a plethora of positive characteristics, but they also have the potential to reduce the expenses that are linked with the operation. According to Msarah et al. 2020, the microbes *B. subtilis* and *B. amyloliquefaciens* are the best candidates for the synthesis of amylase. As a method of protein engineering, the usage of protoplast fusion and transformation procedures for mutagenesis and genetic recombination has been widely utilized by many researchers as a means of achieving strains with higher enzyme productivity or desirable characteristics (Farias et al., 2021).

UV light

The lethal effects of ultraviolet radiation on microorganisms results in DNA harm.The mutagenic impact of non-ionizing radiation, specifically ultraviolet (UV) light, is attributed to the excitation of electrons. The stimulation of electrons within DNA molecules frequently leads to the creation of additional bonds between the neighbouring pyrimidines, particularly thymine, within the DNA structure (Witkin et al., 1996;Kneuttinger et al., 2014). The covalent linkage of two pyrimidines is referred to as a pyrimidine dimer. The formation of dimers frequently induces conformational alterations in cellular DNA and may potentially impede replication processes. The cellular system frequently tries to resolve pyrimidine dimers prior to replication; however, the repair process may also result in genetic alterations (Zhang et al., 2006). UV-A radiation typically results in DNA damage over the generation of oxygen and hydroxyl radicals thenproduce strand DNAand DNA protein crosslinks(Peak and Peak, 1989). On the other hand, the absorption of energy from UV-B radiation results in direct DNA damage. UV-B radiation causes two primary types of lesions, namely the cyclobutane pyrimidine dimer and the pyrimidine-pyrimidine photoproduct. The detection and quantification of these photoproducts can be achieved through the utilisation of specific radio-immunoassays (Mitchell et al., 1985).

UV radiation is mutagenic across a range of organisms. The biological effects, specifically survival and mutation are demonstrated within the wavelength range of 254 to 320 nm. In recent, a study was conducted to enhance the α-amylase productivity of *Bacillus subtilis* TLO3 using UV mutagenesis and selection on the basis to overproduce α-amylase with thermo and alkali stability, which and further used as an additive to detergents (Khelil et al., 2022). Another study aimed to produce amylase from banana (*Musa sapientum*) peels by using mutant *Aspergillus* sp. strains obtained via ultraviolet (UV) radiation and nitrous acid-based mutagenesis (Oshoma et al., 2022).

Chemical mutagens changes in the DNA sequence

Several chemical agents, such as nitrous acids and ethyl methane sulphonate (EMS), are utilised to provoke mutations in bacteria. The application of chemical mutagenesis through N-methyl-N'-nitro-N-nitrosoguanidine (NTG) or UV radiation has been employed to produce strains with enhanced productivity, resulting in a substantial rise in α-amylase synthesis, occasionally reaching up to two or three times the initial quantity (Bajpai et al., 2015; Hungund et al., 2010).

Ribosomal engineering

The emergence of drug-resistant mutations in microorganisms is evidence of changes in the structure and function of the ribosome and RNA polymerase. The alterations exert a substantial impact on the secondary metabolic processes in the mutated strain. Ribosomal engineering is a viable method for generating mutant strains that exhibit increased production of secondary metabolites through the screening of diverse drug-resistant mutants (Singh et al., 2011). α-Amylase production by a strain of *Bacillus subtilis*, was enhanced by the introduction of mutations that produce streptomycin resistance and also increased level of α-amylase production attributed to the increased activity of the ribosomal protein S12 (Kurosawa et al., 2006).

Protoplast fusion technology

Protoplast fusion is a genetic recombination technique. Protoplasts can be generated by exposing cells to wall-degrading enzymes in isotonic solutions, resulting in cells that lack their cell walls.The fusion of cells, followed by the fusion of nuclei, has the potential to occur between protoplasts of different strains that would not typically fuse (Lal et al., 2019; Stanbury et al., 2000). The resulting protoplast fusion may then undergo cellular regeneration, including the growth of a new cell wall, ultimately leading to the development of a normal cell. The fusion of protoplasts has been successfully accomplished in various microorganisms such as filamentous fungi, yeast, streptomycetes, and bacteria. The protoplasts of a strain of *Malbrancheasulfurea* that is resistant to catabolite repression and a mutant strain that exhibits over-production of amylase were subjected to isolation and subsequent fusion through the process of electrofusion. The hybrid yield was measured to be 5×10^{-5}. In another study, amylaseenzymes were produced from protoplast fusions of two *Apergillus* species and examined to be active and stable over abroad range of pH (Salar et al., 2007, Salar et al., 2018).

Genetic recombination through transformation

In genetic recombination, a process called transformation occurs when a recipient bacterium absorbs unencapsulated DNA molecules from a donor bacterium. In 1982, Palvaa discovered a gene that encodes α-amylase in *Bacillus amyloliquefaciens* by using direct shotgun cloning with *B. subtilis* as the host organism. The genome of *B. amyloliquefaciens* was partially digested with a restriction endonuclease, and the resulting 2-5 kb fragments were ligated to plasmid pUB110 (Montor et al., 2017; Singh et al., 2011). The hybrid plasmids were then introduced into proficient *B. subtilis* cells through transformation, and the kanamycin-resistant transformants were screened for α-amylase production. The transformed *B. subtilis* showed a significantly higher level of α-amylase activity compared to the wild-type *B. subtilis* Marburg strain, with an approximately 2500-fold increase in activity, and an approximately 5-fold increase in activity compared to the donor *B. amyloliquefaciens* strain (Palvaaet al.,1982; Nagami et al., 1986).

Physiochemical parameters for amylase production

α-amylase production through SmF and SSF has been extensively investigated, and various physicochemical factors have been identified as significant determinants of the process. These factors include growth medium composition, medium pH, phosphate concentration, inoculum age, temperature, aeration level, and carbon and nitrogen sources (Gigras et al., 2002). While most studies have focused on a limited number of mesophilic fungal species, researchers have made efforts to optimize cultural conditions and isolate high-quality strains for large-scale commercial production. Recent studies have also investigated the impact of additional factors, such as the use of agro-industrial waste as a substrate, the addition of surfactants and oils to enhance enzyme production (Sánchezet al., 2022), and the use of microbial consortia for co-culturing and improved α-amylase production. Additionally, researchers have explored the use of genetic engineering to enhance enzyme production (Rai et al., 2021). Overall, understanding the physicochemical factors that affect α-amylase production is critical for optimizing the process and increasing its efficiency.

Substrate sources

Maltose is the first hydrolyzed product of starch induced by amylase. Maltose is widely considered to be the general inducer molecule for α-amylase in various strains of *Aspergillus oryzae*. Studies have shown that the use of maltose and starch as inducers can result in a significant increase in enzyme activity, with some reports indicating up to a 20-fold increase in *A. oryzae* (NRC 401013) (Gupta et al., 2003). While maltose is the preferred inducer, other carbon sources such as lactose, trehalose, and alpha-methyl-D-glycoside have also been shown to induce α-amylase in some strains. The use of glucose and maltose as substrates for α-amylase production is common, but the use of starch remains popular and widespread (Kathiresan et al., 2006). In addition, various othersugars such as lactose, casitone, fructose, oilseed cakes, and starch processing wastewater have been used for α-amylase production. Wheat bran, an agro-processing byproduct, has also been utilized for the economic production of α-amylase through solid state fermentation (SSF). Liquid surface fermentation (LSF) using wheat bran as a substrate has been reported to produce α-amylase from *Aspergillus fumigatus* and *Clavatiagigantea*. Studies have also demonstrated high α-amylase activities from *A. fumigatus* using alpha-methyl-D-glycoside as a substrate (Rana et al., 2013).

Nitrogen sources

Various organic nitrogen sources have been favouredto produce α-amylase by different microorganisms. Gupta et al. (2003) reported the utilization of yeast extract as a supplementary nitrogen source in the production of α-amylase from *Streptomyces* sp., *Bacillus* sp., and *Halomonas meridian*, resulting in increased productivity. Yeast extract has been used in combination with different nitrogen sources, such as bactopeptone, ammonium sulphate, casein, soybean flour, and meat extract, for various microorganisms. Naidu et al. (2013) found that beef extract, peptone, and corn steep liquor were effective organic nitrogen sources for α-amylase production by bacterial strains. Similarly, *Aspergillus oryzae* exhibited maximum α-amylase production when supplemented with soybean meal and amino acids. Nguyen et al. (2002) reported that different inorganic salts, such as ammonium sulphate, ammonium nitrate, and Vogel salts, were also useful in enhancing the production of α-amylase in fungi. The production of α-amylase has been shown to be influenced by the combination of amino acids and vitamins. The effect of vitamins and amino acids on the augmentation of α-amylase production across different microorganisms is variable and inconclusive. Welkeret al. (1967) found that the presence of glycine resulted in a 300-fold increase in α-amylase production by *Bacillus amyloliquefaciens* through the regulation of pH. The utilization of β-alanine, DL-nor valine, and D-methionine was found to be effective in the synthesis of alkaline amylase by *Bacillus* sp. Gupta et al. (2003) reported that the enzyme yields were appropriate only with asparagine, while the importance of arginine in the production of α-amylase from *Bacillus subtilis* has been extensively noted.

Role of phosphate

Phosphate has a crucial function in regulating the biosynthesis of central metabolites in microorganisms, and it also influencesdevelopment and enzyme production (Kumar et al., 2015; Cardoso et al., 2019). For example, a study by

Pandeyet al. (2001) found that when the phosphate levels exceeded 0.2 M, there was a significant improvement in enzyme production and condition in *Aspergillus oryzae*. Moreover, a deficiency in phosphate levels has a significant impact on cell density and enzyme production in *Bacillus amyloliquefaciens*. The study conducted by Kumar et al. (2015) demonstrated that a low phosphate level led to a decrease in cell density and the complete absence of α-amylase production. On the other hand, elevated levels of phosphate can hinder enzyme production by *Bacillus amyloliquefaciens*. Gupta et al. (2003) reported that high phosphate levels can negatively affect enzyme production. Overall, these studies suggest that maintaining an appropriate level of phosphate is critical for regulating enzyme production and growth in microorganisms.

Role of other ions

The impact of various ions on amylase production by *Aspergillus oryzae* was investigated, including K^+, Na^+, Fe^{2+}, Mn^{2+}, Mo^{2+}, Cl^+, $SO_4{}^{2+}$by Dey et al., 2015. However, it is reported that none of these ions had any effect on amylase production, except for Ca^{2+} which exhibited inhibitory properties. In contrast, the presence of Mg^{2+} was found to be crucial for amylase production, as a 50% decrease in production was observed upon its exclusion from the medium. In *Bacillus* sp., the enzyme production was stimulated by the coordinated presence of Na^+ and Mg^{2+}(Wu et al., 2022). Moreover, the incorporation of zeolites to regulate ammonium ions in *Bacillus amyloliquefaciens* led to a rise in α-amylase production.

pH

The pH of the growth medium is a critical physical parameter that can trigger morphological changes in the organism and influence enzyme secretion (Kumar et al., 2019). The optimal pH range for both growth and enzyme production of Bacillus strains used in the industrial production of bacterial α-amylases through SmF is typically between 6.0 to 7.0 (Kheirallaet al., 2018). Similarly, this pH range has been observed to be optimal for enzyme production via SSF. However, the pH values used in different cases are often not explicitly stated, except for some specific instances such as pH 4.2 and pH 8.0 for *Aspergillus oryzae*,and pH 6.8 for *Bacillus amyloliquefaciens* (Breccia et al., 1998; Ayed et al., 2015). In fungal processes, the buffering capacity of certain constituents present in the media can eliminate the need for pH control. The pH value is an important indicator for the onset and termination of enzyme synthesis (Kumar et al., 2019).

Temperature

Amylase production in fungi is influenced by the temperature at which the organism grows. Most studies on amylase production in fungi have focused on mesophilic species within the temperature range of 25 to 35 °C (Jin et al., 1998; Naidu et al., 2013). For instance, *Aspergillus oryzae* has been found to produce the highest yields of α-amylase at temperatures between 30 and 37 °C.Thermophilic fungi, such as *Thermomonosporafusca* and *Thermomonosporalanuginosus*, have also been reported to produce α-amylase at higher temperatures. It was found that α-amylase production by *T. fusca* occurred at 55 °C, while *T. lanuginosus* was able to produce the enzyme at 50 °C.Compared to fungi, bacteria have a broader temperature range for amylase production (Georis et al., 2000). *Bacillus amyloliquefaciens*, for example, has been shown to produce amylase continuously at 36°C. Additionally, hyperthermophilic bacteria like *Thermococcus profundus* can produce amylase even at temperatures as high as 80 °C (Castro et al., 1993; Kumar et al., 2012; Gupta et al., 2003).Overall, the optimal temperature for amylase production varies depending on the organism and its growing conditions.

Agitation

The rate of mixing and oxygen transfer in fungal fermentation is significantly affected by the intensity of agitation, which in turn affects mycelial morphology and product formation (Xia et al., 2014; Amanullah et al., 2002). However, increasing agitation speed beyond a certain limit can have a negative impact on mycelial growth and enzyme production (Wang et al., 2019). Research suggests that alterations in mycelial morphology resulting from fluctuations in agitation rate may not have a significant impact on enzyme production, as long as the specific growth rate remains consistent. Conventionally, agitation intensities of up to 300 rpm have been used for amylase production from different microorganisms (Koller et al., 2015).

Immobilization for amylase production

The goal of improving amylase production can be achieved through experimental methods or design of experiments (DOE) methodology, which has been further improved by advancements in software (Sagu et al., 2022). In one study, a Box-Behnken design was used to optimize incubation time, pH, and starch substrate for enhancing amylase production by *A. versicolor* (Gopinathet al., 2017). Another study by Ngoh et al. (2016) used a factorial design to extract and identify antioxidative and α-amylase inhibitory peptides from Pinto beans. Encapsulation or entrapment of amylase within alginate beads or similar materials is a potential approach for augmenting its activity and stability, as shown in

Figure 2. Overall, these investigations are significant in optimizing processes involving molecules like glutaraldehyde and predicting the immobilization of a greater quantity of such molecules.

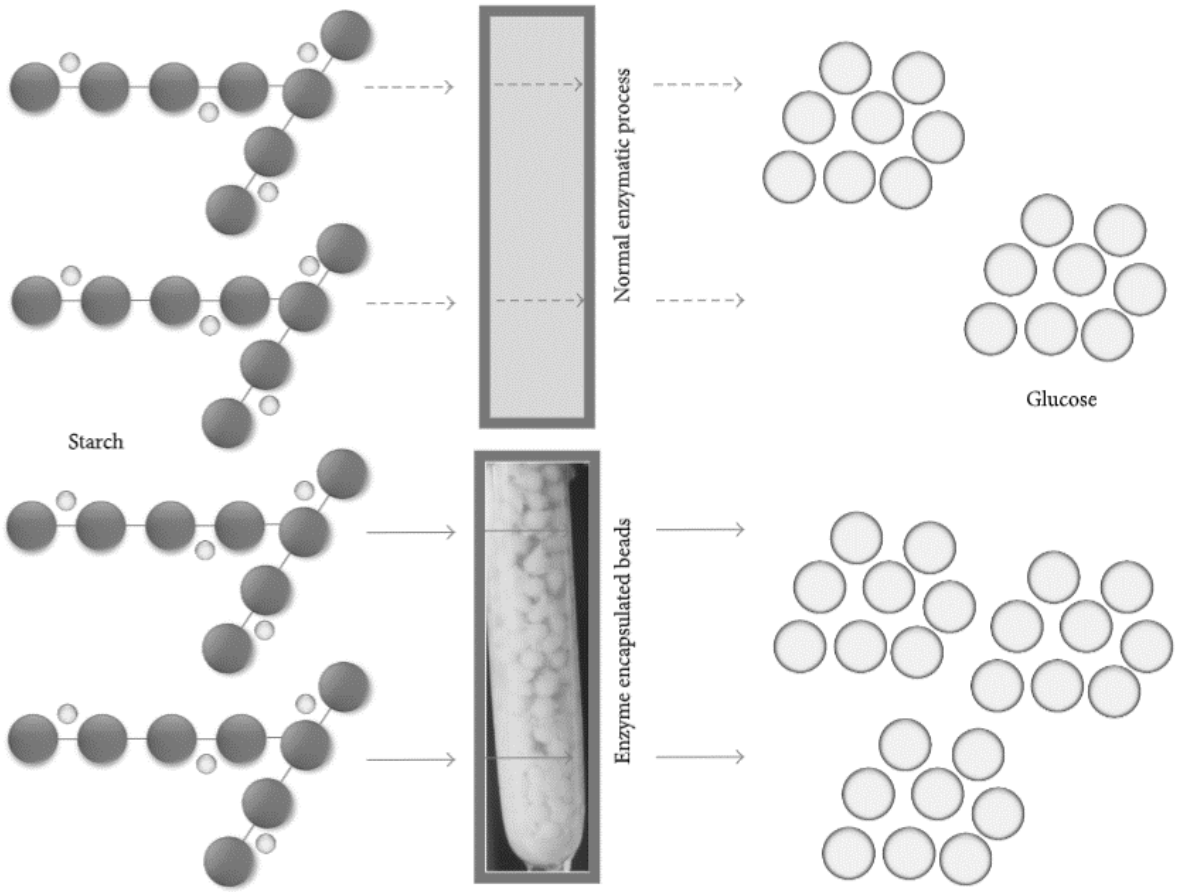

Fig. 2:Increased production of the enzyme amylase. The study conducted by Gopinath et al. (2017) demonstrates variances between the traditional techniques and the utilization of amylase that is encapsulated in alginate beads.

Determination of enzyme activity using the Dinitrosalicylic(DNS) acid method

Enzyme activity is assessed through the quantification of reducing sugars that are generated due to the catalytic action of amylase on starch. An alternative approach involves quantifying the degree of hydrolysis through the analysis of the absorbance of the starch-iodine complex. The dinitrosalicylic acid (DNS) method involves the mixing of aliquots from the substrate stock solution with the enzyme solution. After a 10-minute incubation period at 50 °C, the DNS reagent is introduced into the test tube. The resulting mixture is then subjected to a 5-minute incubation period in a boiling water bath. Upon cooling to room temperature, the supernatant absorbance at a wavelength of 540 nm is quantified. The A540 values for the substrate and enzyme blanks are deducted from the A540 value corresponding to the sample under analysis. In a study on alkalophilic α-amylase from Bacillus strain GM8901, the DNS method was used to determine the enzyme's maximum activity, which was found to be 0.75 U/ml after 24 hours of incubation (Hmidetet al., 2009).

Determination of activity using iodine

The principle underlying the determination of the hydrolytic activity of α-amylase involves the reaction between starch and iodine, which forms a blue-coloured complex. Upon undergoing hydrolysis, the starch complex undergoes a transformation resulting in a final product that exhibits a reddish-browncolor. The measurement of absorbance is typically performed after the end of the enzymatic substrate reaction. The extent of hydrolysis of starch by α-amylase is quantified by this method, as reported by Gupta et al. in 2003.

Dextrinizing activity

An updated version of the assay published by Manning and Campbell (1961) was used to evaluate enzyme activity in a work aimed at characterizing the thermophilic amylase from *Pyrococcusfuriosus*(Wang et al., 2016). The reaction was

stopped by chilling the combination in ice water after the crude enzyme sample had been incubated with soluble starch at 92 °C for 10 minutes. The chromogenic complex formed by the addition of iodine to the reaction mixture with the starch was then diluted with water to produce a distinct spectrum. Using a spectrophotometer, the color intensity was determined at a wavelength of 600 nm (Sundarram et al., 2014; Vogel, 1961).

Purification of amylase

Enzymes are often purified to isolate them for use in various industries. While crude enzyme preparations are suitable for some industrial applications, the clinical and pharmaceutical sectors require extensively purified enzymes (Patel et al., 2023). Purification techniques involve different precipitation, chromatography, and liquid-liquid extraction techniques. Liquid-liquid extraction is an appealing option for purification as it offers several advantages such as reduced viscosity, lower chemical costs, and faster phase separation. This technique has been successfully employed for over a decade for the purification of biomolecules (Gurung et al., 2013). However, a thorough understanding of the behaviour of liquid-liquid extraction systems is necessary to optimize plant-wide control and evaluate potential safety and environmental hazards during the design phase. The properties of the enzyme play a crucial role in determining the effectiveness of the crude extracellular enzyme obtained through fermentation, filtration, and centrifugation. In order to extract intracellular enzymes, raw corn starch is often added to the system, followed by filtration and other relevant procedures (Zaferanloo et al., 2014; Tallapragada et al., 2017). Various precipitating agents, including ammonium sulfate and organic solvents, are commonly used to extract crude enzymes. Additional separation and purification can be achieved through various chromatographic techniques, including ion exchange, gel filtration, and affinity chromatography (Mohamed et al., 2011; Gurung et al., 2013). The purification methods employed for enzymes can vary depending on the species and the specific techniques utilized.

Ezeji and Bahl (2006) documented the precipitation of *Aspergillus falvus var. columnaris* enzyme through the precipitation agents, which was subsequently followed by dialysis and column chromatography. The investigation of the minimal amylase production in *Preussia* involved trichloroacetic acid (TCA)/acetone precipitation and Sephadex G-200 gel filtration column analysis. The fractions were subjected to DEAE-Sepharose ion exchange chromatography to purify and characterize extracellular α-amylase derived from *Clostridium perfringens*, as investigated by Sundarram et al. (2014). The purification of α-amylase from a mutated Bacillus subtilis strain was outlined by Yang et al. (2020) through a series of sequential procedures. The methods employed in the study encompassed precipitation utilizing 80% ammonium sulfate, ultrafiltration, dialysis, and Sepharose column chromatography. Thermotolerant amylases can be obtained by subjecting the cell extract, which has been obtained through centrifugation and elevated temperatures for reducing cell debris and to denature thermolabile proteins. The purification of amylase produced by *Thermotogamaritima* MSB8 was carried out using anion exchange chromatography. he selection of purification methods is dependent on the specific properties of the enzyme and the species from which the enzyme was derived (Liebl et al., 1997; Damián et al., 2008; Zhang et al., 2019).

CONCLUSION

Amylases are enzymes that can break down starch into smaller sugars through hydrolysis. The use of microorganisms to produce amylase is becoming more common in the starch processing industry due to their cost-effectiveness and technical advantages. These enzymes have diverse applications in the food industry, including producing glucose syrups and preventing haze in juices. They are also used in various other industries, including baking, paper, textile, and detergent. Researchers are currently focusing on discovering new amylases that exhibit improved properties such as enhanced stability at high pH and temperature, higher catalytic efficiency, better starch gelatinization, reduced viscosity, and increased resistance to microbial contamination. The ultimate goal of these investigations is to expand the potential uses of amylases in different industries.

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