International Journal of Advanced Research in Biological Sciences
ISSN: 2348-8069
www.ijarbs.com
(A Peer Reviewed, Referred, Indexed and Open Access Journal)
DOI: 10.22192/ijarbs Coden: IJARQG (USA) Volume 11, Issue 1-2024

Review Article

DOI: http://dx.doi.org/10.22192/ijarbs.2024.11.01.008

A Mini Review on "Exploring the Potential of -Amylase: Genetic Identification, Characterization, and Insights into Production and Purification Techniques"

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Abstract

-Amylase is the main enzyme widely employed in various industrial purposes, including foodstuffs, fabric goods, and biofuel production. In our review study, we focused on genetically identified and characterized -amylase producing microbial isolates from diverse sources. The 16S rRNA gene was amplified from bacterial cultures using an alkaline lysis protocol and heat shock technique for DNA isolation. Following the isolation of DNA samples, the 16S rRNA gene underwent PCR amplification, and later phylogenetic relationship was determined by its sequencing and subsequent analysis. To optimize the enzyme production, various parameters like pH, temperature, incubation period, etc. were investigated. To purify the -amylase enzyme, various techniques were employed. The crude amylase enzyme preparation was precipitated using ammonium sulfate. After additional dialysis purification and size exclusion chromatography separation, the precipitate was processed for further analysis. For the purpose of assessing purity, the dialyzed samples were analyzed using reverse-phase high-performance liquid chromatography (RP-HPLC).Characterization of the -amylase enzyme included determining its molecular weight. SDS-PAGE, gel filtration chromatography, and mass spectrometry techniques were employed for this purpose. In conclusion, this study we successfully compared the genetically identified and characterized -amylase producing microbial isolates. The optimized incubation duration and purification techniques provided valuable insights into the production and purification of this industrially important enzyme. Further studies can focus on enhancing the enzyme's characteristics and exploring its potential applications in various industries.

Keywords: -amylase, phylogenetic analysis, SDS-PAGE, RP-HPLC, purification.

Introduction

Enzymes are crucial for sustaining life on Earth, with -amylase being the most precious enzyme for various industries. -amylase, a member of the glycoside hydrolase family (GH13), is commonly derived from microbial sources[1]. It acts on starch and similar carbohydrates, breaking down -(1,4)-glucosidic bonds to let out glucose and malto-oligosaccharides[2]. This enzyme is extensively used in sectors such as food production, textiles, paper manufacturing, detergents, beer brewing, and medical equipment [3]. It constitutes a remarkable portion (25-30%) of universal enzyme usage. While -amylases can be found in plants, animals, and microbes, fungi and bacteria are preferred for factory manufacture due to their reliability, adaptability, and of low cost. These microorganisms offer advantages in terms of production efficiency, requiring less money, time, and space[4]. The bioprocess approach is favoured for amylase production due to its simplicity, affordability, speed, and the availability of suitable enzyme variants [5]. Recent industry focus has been on developing amylases with enhanced characteristics, examples of desirable characteristics include enhanced functionality, specificity towards substrates, heightened thermal resilience, adaptability to a broad pH spectrum, and resistance to denaturing agents and heavy metals. Consequently, research efforts have been directed towards isolating and improving novel amylase-forming *Bacillus* strains fit for various Commercial utilization[6]. However, there is still spa for refinement. For example, the production of amylases with high optimum temperatures often requires specialized process designs and additional energy consumption [7,8]. Therefore, there is a demand for screening mesophilic strains that produce amylase with high thermostability and at elevated temperatures. The cost of amylase production accounts for a significant portion (24%) of the overall process cost [9], leading to increased interest in finding ways to reduce the price by increasing enzyme yield and/or activity.

Types of Amylase

-Amylase

There are different types of amylase enzymes with distinct functions and characteristics. One such type is -amylase (E.C.3.2.1.1), which causes the cleavage of -1,4-glycosidic linkages in various carbohydrates. This enzyme is environmentally safe and enhances the effectiveness of detergents in removing tough stains. Researchers are motivated to explore the best, safest, and most practical methods of producing -amylase due to its numerous applications [6].

– Amylase

Another type is -amylase (EC 3.2.1.2), which cleaves -1,4-glucan bonds. It acts from the nonreducing edge of a polysaccharide chain, generating successive maltose monomeric units. Nevertheless,it lacks the ability to cleave the branching linkages present in branched polysaccharides such as glycogen or amylopectin, resulting in the persistence of dextrin units. -amylase is mainly sourced from yams and the higher plantseeds. It exhibits optimal activity within a narrow pH range of 4.0 to 5.5 [10].

– Amylase

The third type is -amylase (EC 3.2.1.3), which differs from other amylases in its ability to cleave (1-6) glycosidic linkage as well as the terminal (1-4) glycosidic linkages at the non-reducing terminus of linear amylose and branched amylopectin, releasing glucose units. -amylase works most under acidic mediums, with an optimum pH of 3 [10].

In summary, these different types of amylase enzymes have specific roles and exhibit variations in their substrate specificity, pH requirements, and cleavage patterns.

-Amylase Production

Chief Sources

-Amylase can be obtained from various sources, including plants, animals, and microbes. In current years, there has been an increasing focus on microbial production of -amylase. Microorganisms are becoming increasingly popular as a source due to two main reasons. Firstly, microorganisms have a rapid multiplication rate, which allows for quick and scalable production of the enzyme. Compared to animals and plants, microorganisms are easier to handle and require less space and financial resources. Secondly, microorganisms can be easily modified through genetic engineering and other techniques. This enables strain improvement, mutation, and other modifications to optimize -amylase production. Microbes can be engineered to meet the specific requirements of expanding manufacturing sectors and to produce enzymes with properties of interest, such as broad range temperature stability. Thermostable -amylases are preferred as they reduce the chances of contamination, accelerate reactions, and save energy. Additionally, conducting the cleavage at an elevated temperatures decreases the polymerization of D-glucose to iso-maltose [11].

Genetic Characterization of amylase producing isolates

To genetically identify -amylase producing strains obtained from various sources, amplification of 16S rRNA gene was carried out. DNA was isolated from bacterial cultures using the alkaline lysis protocol and heat shock technique. Universal primers targeting the sequence of 16S rRNA gene were employed for PCR amplification, following the specific conditions of 30 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes. After the finish of amplification process, a portion of the PCR mixture was analyzed by employing horizontal gel electrophoresis, while the leftover mixture was purified by employing QIAquick PCR purification reagent. The PCR product was then subjected to sequencing. The resulting fragments of the 16S rRNA gene sequence were compared with the NCBI database using the nucleotide BLAST tool to determine their closest taxonomic relatives. To visualize the phylogenetic relationships, a method named as neighbor-joining was employed to make a phylogenetic tree using the NCBI distance tree [30]. Additionally, Km and Vmax, which are kinetic parameters, were calculated using the Lineweaver-Burk plot as shown in table 1.

Production Processes

When producing -Amylase on a large scale for use in industry, there are primarily two techniques used. These are (1) Solid State Fermentation and (2) Submerged Fermentation. The enzyme -Amylase has been produced using both of these techniques.

Process Parameters

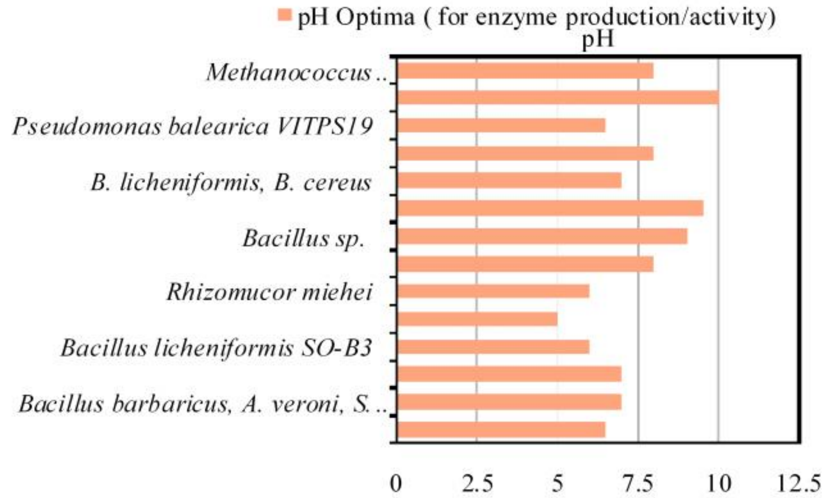
On the basis of microbiological source, final product of interest, technique of fermentation used, and several other similar considerations, different process control parameters are Optimized and their results have been shown in figure 1.

Table 1: Amylase-producing microorganisms along with Primers pair used for amplification of 16S rDNA gene and other process parameters.

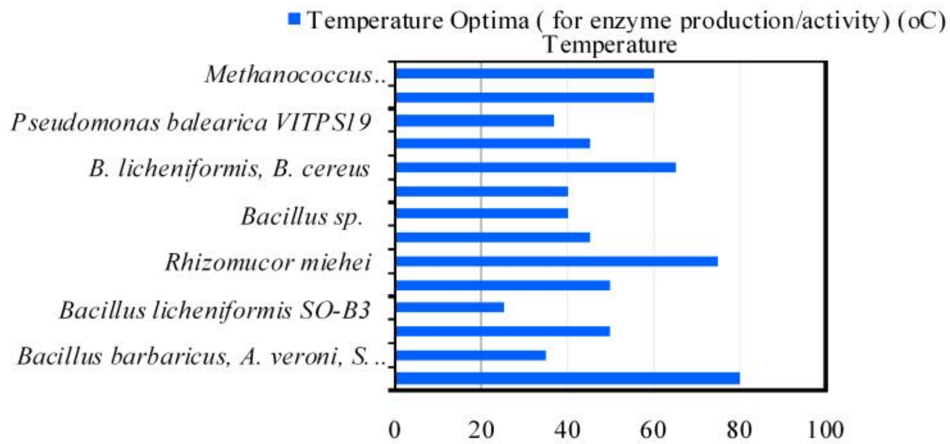
S.No.	Microorganisms	Molecular identification (16S rDNA gene sequencing method)	Primers set used for 16S rDNA gene amplification	Technique used for molecular weight determination	Molecular weight of Amylase (kDa)	Km	Vmax	References
1	*M. vulcanus, Haloarcula* sp.	Yes	21F (5'-TTCCGGTTGATCCTGCCGGA-3') 1492R (5'- GGTTACCTTGTTACGACTT-3')	LC-MS/MS	43.70 70.16 60.02	-	-	[12]
2	*Pseudomonas balearica VITPS19*	-	-	SDS-PAGE	47	45.23 mM	20.83 U/mL	[14]
3	*B. licheniformis, B. cereus*	Yes	Forward5'-AGAGTTTGATCCTGGCTCAG-3' Reverse 5'-ACGGCTACCTTGTTACGACTT-3'	-	-	-	-	[16]
4	*Bacillus megaterium*	Yes	Forward 5'-AGA GTT TGA TCM TGG CTC AG - 3'. Reverse 5'- AAG GAG GTG ATC CAN CCR CA - 3'	-	-	-	-	[17]
5	*Bacillus* sp.	Yes	fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') rP2 (5'-ACGGCTACCTTGTTACGACTT- 3')	-	-	-	-	[18]
6	*Bacillus* sp. FW2	Yes	518F (5'-CCA GCA GCC GCG GTA ATA CG-3') 800R (5'-TAC CAG GGT ATC TAA TCC-3')	SDS-PAGE	55	-	-	[19]
7	*Rhizomucor miehei*	-	-	SDS-PAGE	49.9	-	-	[20]
8	*B. subtilis* S113	Yes	Forward5'-AGA GTT TGA TCC TGG CTC AG-3'. Reverse5'-ACG GCT ACC TTG TTA CGA CTT-3'	SDS-PAGE	66	0.22%	357.14 U/mg	[21]
9	*Bacillus licheniformis* SO-B3	Yes	-	SDS-PAGE	74	0.005 mM	3.5 μmol min−1	[22]
10	*Bacillus mojavensis* UMF29	Yes	-	-	-	111 mg/ml	526 U/ml	[23]
11	*B. barbaricus, A. veroni, S. maltophilia,*	Yes	Forward. 5'- ACGGGCGGTGTGTAC -3' Reverse 5'-CAGCCGCGGTAATAC-3'	SDS-PAGE	19 56 49	-	-	[5]
12	*Trichoderma harzianum*	-	-	-	-	1.2–3.2 mg/ml	-	[24]
13	*B. atrophaeus*	Yes	Forward 5'- AGAGTTTGATCMTGGCTCAG- 3' Reverse 5'-TACGGYTACCTTGTTACGACTT-3'	SDS-PAGE	61	0.72 mg/ml	667 U/ml	[1]
14	*B. subtilis Y25*	Yes	27f (5'- AGAGTTTGATCMTGGCTCAG-3') 1492R (5'-ACCTTGTTACGACTT-3')	Gel Filtration	58	314.10 ± 23.30 Units/mg	53.98 ± 12.03 mg/ml	[4]
15	*Bacillus sp. WangLB*	Yes	HAD-1 (5'- GACTCCTACGGGAGGCAGCAGT-3') E1115R (5'-AGGGTTGCGCTCGTTGCGGG -3')	SDS-PAGE	55	0.37 mg/ml	233 U/mg	[3]
16	*L. manihotivorans* LMG 18010T	-	-	SDS-PAGE	135	3.44 mg/ml	0.45 mg hydrolysed starch/ml/ min	[27]
17	*B. licheniformis* AI20	Yes	Forward(5'-AGAGTTTGATCMTGGCTCAG-3') Reverse(5'-TACGGYACCTTGTTACGACTT-3')	SDS-PAGE	55	0.709 mg/mL	454 mU/mg	[28]
18	*Chromohalobacter sp.* TVSP 101	-	-	SDS-PAGE	72 62	125mM 166 mM	5.88U/mg 5.0 U/mg	[29]
19	*Bacillus velezensis* KB 2216	Yes	Forward(5'-AGAGTTTGATCCTGGCTCAG-3') Reverse1492R (5'-TACCTTGTTACGACTT-3')	SDS-PAGE	97	0.20 mg/ml	226.3 U/mg/min	[30]
20	*Geobacillus icigianus* BITSNS038	Yes		SDS-PAGE	45-66	2.17 mg/mL	4.16 U/mL	[31]

Table 1

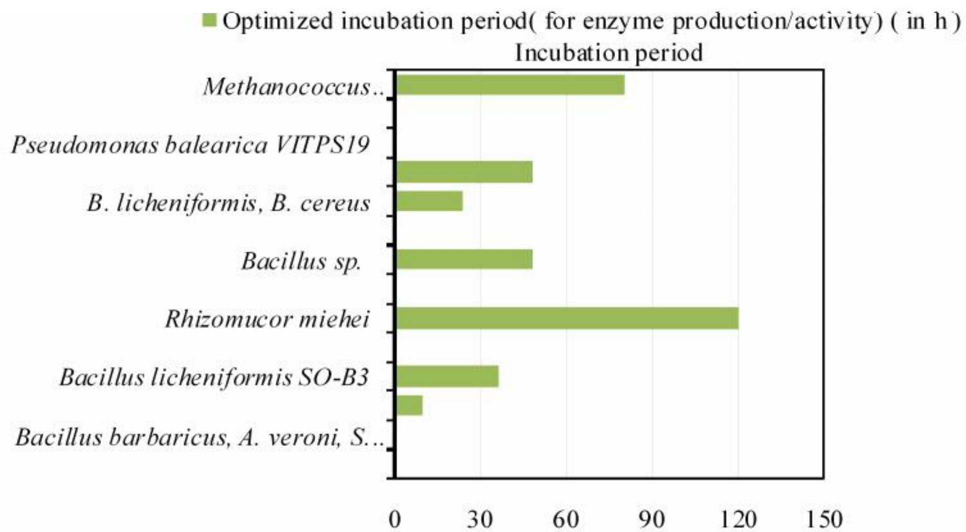
A



B



C



pH

The optimal pH is a crucial parameter for maintaining the stability and activity of -amylase during the production process. Enzymes are sensitive to pH, so it is important to carefully control the pH during production. Our analysis revealed that *Methanococcus vulcanus* and *B. subtilis S113*, two bacterial strains, have an optimum pH of 5 for -amylase production. On the other hand, fungi such as *Rhizomucor miehei* and *Trichoderma harzianum* have pH optima of 6 and 6.5, respectively, for -amylase activity. Additionally, bacterial strains like *Bacillus licheniformis SO-B3*, *Bacillus mojavensis* UMF29, and *Bacillus megaterium* have pH maximum falling in a range from 6 to 6.5 and 7 for -amylase production the results have been shown in panel A in fig.1.

Temperature

Temperature plays a crucial role in determining the optimal activity of enzymes. Since enzymes are sensitive to temperature, it is important to carefully control the temperature during the production process. Our research review revealed that *Bacillus* sp. and nine other bacterial strains, such as *Pseudomonas sp*., exhibit optimal temperature ranges between 40 and 60 degrees Celsius. Similarly, for the fungi *Rhizomucor miehei* and *Trichoderma harzianum*, the ideal temperature ranges were found to be 50 and 80 degrees Celsius, respectivelythe results have been shown in panel B in fig.1.

Incubation period

In this review, we identified the incubation duration as a critical factor for the production of enzymes from microbial cultures. Our findings revealed that one *Bacillus species* demonstrated an optimal incubation period of 24 hours for amylase generation. *Bacillus licheniformis* SO-B3 exhibited an ideal incubation time of 36 hours for amylase synthesis. Additionally, two bacterial strains showed an optimal incubation time of 48 hours for amylase production. Interestingly, we observed that the *Rhizomucor miehei* fungal strain had the longest optimal incubation period for amylase synthesis the results have been shown in panel C in fig.1.

Determination of enzyme activity

Here, we will discuss some commonly used techniques for measuring enzyme activity. These methods include quantifying the amount of reducing sugars produced by -Amylase during starch hydrolysis and analyzing the absorbance of the starch-iodine complex to assess the extent of hydrolysis.

DNS (Dinitrosalicylic Acid Method)

In the dinitrosalicylic acid technique, a portion of the stock solution of substrate is combined with the enzyme solution. The DNS reagent is then poured to the test tube, and incubatethe mixture at 50°C for 10 minutes. Afterward, it is further put in a boiling water bath for 5 minutes. The absorbance value of the supernatant is obtained at 540 nm after cooling to room temperature. To calculate the A540 value for the tested sample, the absorbable value at 540 nm for the substrate blank and the absorbable value at 540 nm for the enzyme are subtracted [13-16,18-20,22,24].

Determination of Activity Using Iodine

The formation of a blue-colored complex between starch and iodine can be utilized to estimate the hydrolytic activity of -amylase. As starch is hydrolyzed, this complex undergoes a colour change to reddish brown. Once the enzyme-substrate reaction is complete, the absorbance can be measured, providing a quantification of the extent to which -amylase has hydrolyzed starch [12,17,21].

Purification of -Amylase

Enzymes used in manufacturing applications are often prepared in a crude manner, requiring less post-processing. However, in the pharmaceutical and medical industries, highly pure enzymes are necessary. Purification techniques such as liquid-liquid extraction, chromatography, and

precipitation are commonly employed, with a merger of these methods used in a sequential manner to attain themaximum purity. The level of purity desired determines the number of purification steps required [25]. Initially Filtration is done before the centrifugation employed to separate crude extracellular enzyme samples from the fermented mass. Concentration of the crude amylase enzyme can be achieved through ammonium sulfate precipitation method or organic solvents. Further concentration can be achieved by dialysis made against double distilled water or a buffer solution. Chromatographic techniques e.g. ion exchange, gel filtration, and affinity chromatography can be employed to separate and purify the enzyme [26].

In a specific case involving the purification of the enzyme generated by *Haloarcula* sp. HS, the fractions with starch-degrading activity viz. the concentrated supernatant and partially purified cell extract were subjected to dialysis for 48 hours to remove excess salt in these fractions. The proteins were initially precipitated, followed by treatment with dithiothreitol. Tryptic digested protein fragments were then analyzed using LC-MS/MS in a triple quadrupole-TOF system coupled with a nano-HPLC and a nano-electrospray ion source. The Protein Pilot program and the Paragon algorithm were used for peptide and protein identification [12].

Another study focused on the crude amylase enzyme preparation from *Pseudomonas balearica* VITPS19, which was precipitated using ammonium sulfate. After additional dialysis purification and size exclusion chromatography separation, the precipitate was processed for further analysis. The dialyzed samples were subjected to a pre-equilibration step using the same buffer and then loaded on to a Sephadex G50 column. The amylase activity of the obtained fractions was evaluated, and the active fractions were combined. To determine the purity, the combined fractions were eluted out by employing reverse-phase high-performance liquid chromatography (RP-HPLC) [14].

Characterization of -Amylase

Determination of molecular weight/mass

After purifying the -amylase, various techniques including SDS-PAGE, Gel Filtration chromatography, and Mass spectrometry were used to characterize the enzyme and predict its molecular weight. In one study, a *Bacillus* sp. strain was found to produce amylase. The TCA/acetone protein precipitation technique was employed to separate and purify the enzyme. A single band was shown on SDS-PAGE analysiswith a molecular weight of 55 kDa for the purified amylase. According to literature, the molecular weight of -amylase made by diverse *Bacillus* spp. ranged from 21 to 150 kDa. Interestingly, the -amylase synthesized by *Bacillus* sp. strain Wang LB shared the same molecular weight as *B. licheniformis* AI20 and *B. licheniformis* SKB4 [25]. Another investigation determined the molecular mass of pure -amylase from *Bacillus licheniformis* SO-B3 to be 74 kDa. The literature reports variations in the molecular mass of -amylase. For instance, *Bacillus mojavensis* SO-10 was reported to have a molecular mass of 73 kDa, *Anoxybacillus flavithermus* had a molecular mass of 60 kDa, and *Geobacillus stearothermophilus* had a molecular mass of 63 kDa. *Bacillus cereus* had a molecular mass of 55 kDa, *Bacillus subtilis* had a molecular weight of 56 kDa, *Alicyclobacillus sp.* A4 had a molecular weight of 64 kDa, and *Anoxybacillus flavithermus* sp. SO-19 had a molecular mass of 96 kDa [22]. In other investigations, the molecular mass of -amylase produced by *L. manihotivorans* LMG 18010T, *B. licheniformis* AI20, *Chromohalobacter* sp. TVSP 101, *Bacillus velezensis* KB 2216, and *Geobacillus icigianus* BITSNS038 were found to be 135 kDa, 55 kDa, 72 kDa, 62 kDa, 97 kDa, and 45-66 kDa, respectively [27-31]. Therefore, it can be concluded that the purified enzyme is -amylase**.**

Table 2 : Amylase-producing microorganisms along with the substrate and optimized process parameters

S.No.	Microorganisms	Substrates/media	Amylase assay/protein determination	pH Optima (for enzyme production/ activity)	Temperature Optima (for enzyme production/ activity) (oC)	Optimized incubation period(for enzyme production/ activity) (in h)	Optimization method	References
1	*Methanococcus vulcanus, Haloarcula* sp.	Synthetic media	Standard iodine assay	-/5 - 9	-/60	-/80	OFAT	[12]
2	*Lysinibacillus xylanilyticus*	Synthetic media	Bernfeld	-/8-10	60	-	OFAT	[13]
3	*Pseudomonas balearica VITPS19*	Synthetic media	Bernfeld/Bradford	7/6.5	28-37	-	OFAT,RSM	[14]
4	*Bacillus* sp. strain RU1	Synthetic media	Bernfeld	6-9/-	-/45	48	OFAT	[15]
5	*B. licheniformis* *B. cereus*	Synthetic media	Bernfeld/Lowery	6-7	45-65	24	OFAT	[16]
6	*Bacillus megaterium*	Synthetic media	Starch iodine method	6.0-9.5	40	-	OFAT	[17]
7	*Bacillus* sp.	Synthetic media	Bernfeld	6-9	40	48	OFAT	[18]
8	*Bacillus* sp. FW2	Synthetic media	Bernfeld	7-8	40-45	-	OFAT	[19]
9	*Rhizomucor miehei*	Synthetic media	Bernfeld/Lowery	6	75	120	OFAT	[20]
10	*B. subtilis* S113	Synthetic media	Starch iodine method	5	50	-	OFAT	[21]
11	*Bacillus licheniformis* SO-B3	Synthetic media	Bernfeld	6	25-40	36	OFAT	[22]
12	*Bacillus mojavensis* UMF29	Synthetic media	Bernfeld	7	40 & 50	2-10	OFAT	[23]
13	*Bacillus barbaricus, Aeromonas veroni,* and	Synthetic media	Sharma /Lowery	7	35 & 50	-	OFAT	[5]
14	*Trichoderma harzianum*	-	Bernfeld	6.0 & 6.5	50 & 80	-	OFAT	[24]

Conclusion

Enzymes are highly susceptible to unfavourable conditions. In this review, we explored different approaches for the production of -amylase from diverse microbial sources. -Amylase, a widely utilized enzyme, offers versatile applications in various industries. Currently, there is a significant abundance of agricultural and industrial waste that can serve as alternative substrates for microbial cultures and enzyme production. Our ongoing research focuses on identifying microbial strains capable of thriving and producing enzymes under challenging culture conditions, diverse production media, wide pH and temperature ranges, and other demanding parameters. The industrial demand for -amylase necessitates the development of enzymes that can function effectively in adverse conditions. By optimizing various parameters, we aim to maximise the practical efficiency of amylases.

Conflict of Interest

There are no conflicts of interest declared by the authors.

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How to cite this article:
Sharma Arvind, Sharma R.K. (2024). A Mini Review on "Exploring the Potential of -Amylase: Genetic Identification, Characterization, and Insights into Production and Purification Techniques". Int. J. Adv. Res. Biol. Sci. 11(1): 71-81.
DOI: http://dx.doi.org/10.22192/ijarbs.2024.11.01.008