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REVEALING THE POTENTIAL OF *AZADIRACHTA INDICA* A. JUSS. FOR THE INDUCTION OF AMYLOLYTIC AND PULLULYTIC ENZYMES

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Abstract

In vitro culture of *Azadirachta indica* offers controlled and constant provision of metabolites and therefore could be more feasible in the enzyme induction study. In this research, we developed cell suspension cultures through neem callogenesis and incorporated different percentages of inducers, namely starch, dextrin, and pullulan in the suspension media to analyze their effects on the induction of amylolytic and pullulytic enzymes. The cell-free extracts of the induced cultures were analyzed on starch and pullulan agar-solidified plates for the zones of hydrolysis. Additionally, enzyme activities were quantified using the DNS method. Consequently, starch and pullulan degrading activities were detected in the cultured media, confirming the successful induction of amylolytic and pullulytic enzymes. These enzymes hold significant commercial value in the food, textile, and detergent industries. This research represents the first report in the literature on pullulan degradation by neem enzymes and offers valuable insights for future research in this area.

Key words: *Azadirachta indica,* Plant enzymes, Neem tissue culture, Amylolytic enzymes, Pullulytic enzymes.

Introduction

Azadirachta indica A. Juss., commonly known as "Neem", is a versatile tree. The word neem means "Bestower of good health." It is due to its never-ending benefits to humanity that it has been recognized as one of the most promising trees of the 21st century (WHO/UNEP, 1989) and as "A tree for solving global problems" (Anon., 1992). Neem possesses valuable applications in agriculture, medicine, and environmental protection, and possesses a wide spectrum of biological activities. (Koul & Wahab, 2007; Nicoletti & Murugan, 2013).

Neem appears to be a biochemical factory of more than hundreds of biologically active compounds (Saleem *et al.*, 2018). Almost all parts of neem, such as leaf stem, bark, gum, root, flower fruit and seed are beneficial and commercially exploitable (Brahmachari, 2004). Studies have evaluated the potential of neem for producing hydrolytic enzymes through various extraction and fermentation methods. Protease enzymes have been extracted from neem leaves using an aqueous two-phase extraction process (Gupta & Chaphalkar, 2015). Additionally, neem oil seed cake has been used as a substrate for producing lipase enzymes through solid-state fermentation (Manjula *et al*., 2017).

Further research into neem callogenesis for hydrolytic enzyme production has demonstrated that incorporating exogenous substrates, namely, starch and casein, into cell suspension media induced the expression of amylase and protease respectively. Dextrin supplementation also led to hydrolytic activity (Warisha *et al*., 2019). However, this dextrin degradation in the previous study may be attributed to either amylase or limit dextrinase (LD) in the suspension media. LD, also known in the literature as Pullulanase, pullulan 6-glucanohydrolase or R-enzyme, is an enzyme with the systematic name dextrin 6-alpha-glucanohydrolase. It catalyzes the hydrolysis of ∝-1,6 glycosidic bonds found in ∝- and β- limit dextrins, amylopectin, and pullulan.

Unlike the previous research that focused primarily on enzyme production, this study investigates the effects of varying concentrations of inducers, such as dextrin, pullulan, and starch on enzyme production in cell suspension media. Through qualitative and quantitative analysis, it aims to assess amylolytic enzyme production and identify pullulan-degrading activity in the cultured media. Since starch, pullulan, and glycogen serve as important energy sources for plants, animals, and microbes (Vihinen & Mantsala, 1989), it is hypothesized that similar to starch and dextrin degradation, neem callus cells may have the potential to produce pullulytic enzymes capable of pullulan degradation.

In the present study, the induction of amylolytic and pullulytic enzymes occurred in neem cell suspension cultures in the presence of specific enzyme inducers, proper nutrients, and signaling molecules (plant growth hormones). The utilization of cell suspension culture in enzyme induction not only stimulates increased enzyme production in response to an exogenous substrate but also provides deeper insights into the enzymatic potential of the plant cells. Enzyme induction refers to an increased enzyme synthesis (increased quantity) when exposed to an exogenous substance (Alsanosi *et al*., 2014). The exogenous substances (inducers) are used to stimulate enzyme production in cultured media as reduced enzyme yield could impede the progress of the study. Among various enzyme induction methods, chemical induction is the most effective, utilizing specific chemical compounds to stimulate enzyme production, leading to significantly higher yields in cultured cells (Kedderis, 2010).

Amylolytic enzymes: The amylolytic enzymes, generally called amylases, hydrolyze starch and other related polymers such as glycogen, pullulan and other oligo and polysaccharides (Vihinen & Mantsala, 1989; Pandey *et al*., 2000; Janeček, 2009). Starch is a complex polysaccharide consisting of two different fractions: (1) branchless amylose, a linear polymer of glucose units joined by α-1,4 glycosidic bonds, and (2) amylopectin, which consists of α-1,4 linked glucose chains with additional branching

through α-1,6 glycosidic bonds (Ball *et al*., 1996). A combination of four enzyme groups, namely endoamylases, exoamylases, debranching enzymes, and transferases, leads to complete starch degradation (Guzmán-Maldonado & Paredes-López, 1995).

Both endoamylases and exoamylases have affinities for α-1,4-linkages in starch and related polysaccharides. The popular exoamylases are *α*-glucosidase and glucoamylase, which act on both *α*-1,4 and *α*-1,6 glycosidic bonds, while *β*-amylase specifically hydrolyzes *α*-1,4 bonds from the non-reducing-ends of the polysaccharide. The α-amylases and glucoamylases are produced by a diverse range of microorganisms (Fogarty, 1983; Vihinen & Mantsala, 1989), animals, and plants (Janeek & Bala, 1992; Pandey *et al.*, 2000). In contrast, β-amylases are predominantly present in higher plants and some microorganisms (Adachi *et al*., 1998; Mikami *et al*., 1999). Amylases account for approximately 30% of the global enzyme production (Vaidya *et al*., 2015). The debranching enzymes specifically target α-1,6-bonds in amylopectin within starch, as well as in pullulan, related polysaccharides, and dextrins. Pullulanase and isoamylase are direct-acting debranching enzymes found in a wide range of plants and microorganisms. These enzymes have significant commercial value and are extensively utilized in the food, textiles, fermentation, detergent, and paper industries (Pandey *et al*., 2000).

Pullulytic enzymes: Pullulytic enzymes, also known as pullulanases, specifically hydrolyze α-1,6-glucosidic bonds in pullulan and other branched polysaccharides including starch. Pullulan is a polysaccharide consisting of repeating maltotriose units, where each unit contains three glucose molecules linked by α-1,4-glycosidic bonds, while successive units are connected through α-1,6-glycosidic linkages. Pullulan serves as a standard substrate for identifying starch-debranching enzymes such as pullulanase (Haki & Rakshit, 2003).

Literature often uses the terms limit dextrinase and pullulanase interchangeably; however, pullulanase is more commonly associated with the debranching enzymes of plant origin (Morinaga *et al.*, 1997; Francisco *et al*., 1998; Cho *et al*., 1999; Beatty *et al*., 1999; Wu *et al*., 2002). Naturally, starch breakdown in plants requires the synergistic action of pullulanase along with α and β-amylases for complete starch breakdown. However, pullulanase also plays a crucial role in starch biosynthesis by influencing the structural arrangement of amylopectin (Pan & Nelson, 1984; Mouille *et al.*, 1996; Nakamura *et al*., 1997; Kubo *et al*., 1999; James *et al*., 2003). Plant pullulanase readily hydrolyzes the α-1,6-glucosidic bonds in pullulan, β-limit dextrin, and α-limit dextrin, but its debranching activity on amylopectin is relatively lower compared to other polyglucans (Drummond *et al.*, 1970; Lee *et al*., 1971; Dunn *et al*., 1973; Gordon *et al*., 1975; Yamada & Izawa, 1979; Okita & Preiss, 1980; Li *et al*., 1992). Unlike pullulanase, isoamylase does not hydrolyze pullulan (Van Der Maarel *et al*., 2002). Previous studies have demonstrated the presence of pullulanase activity in a diverse range of plant species. These include broad bean (Gordon *et al*., 1975), sorghum (Hardie *et al*., 1976), oat (Yamada, 1981), pea (Zhu *et al*., 1998), wheat (Netrphan, 2002), maize (Dinges *et al.*, 2003), rice (Yamasaki *et al*., 2008), barley (Peng & Hu, 2012), sunflower (El-Shora *et al*., 2015), and cassava (Wangpaiboon *et al.*, 2023).The optimum pH of the enzyme is similar across many plant species (5.0-5.6); however, the optimum temperature varies among different plants.

Pullulanase is essential for producing high-glucose and high-maltose syrups through starch saccharification because α-amylase and β-amylase have little to no activity towards α-1,6 linkages of starch. Therefore, a debranching enzyme like pullulanase is required for complete starch lysis (Naik *et al*., 2023). Alkaline pullulanases have found great application in dishwashing and laundry detergents for effective starch breakdown under alkaline pH (Hii *et al*., 2012). Verified Market Research (2024) reported that the global pullulanase market was valued at USD 161.8 million in 2023 and is expected to grow to USD 391.76 million by 2030, indicating its increasing industrial demand.

Material and Methods

Media preparation: Callus induction and maintenance were performed using Murashige & Skoog (MS) medium. (Murashige & Skoog, 1962). The MS medium was supplemented with 4 mg/L indole-3-butyric acid (IBA) and 1 mg/L benzyl adenine (BA) as growth regulators (Kearney *et al.*, 1994). Stock solutions including 10X macro salts, 100 X micro salts, vitamin solution, and iron-EDTA along with 1 N NaOH, HCL, and growth regulators at 20 mg/ml were prepared and stored at 4°C. The medium contained 0.3% sucrose as the carbon source and 0.9% solidifying agent. The pH of the medium was adjusted to 5.85 before gelling and autoclaving at 121°C, 15 psi for 15 minutes (without a drying cycle).

Explant collection: Disease-free young leaves were obtained from a neem tree near the Biotechnology Department, University of Karachi.

Surface sterilization of explant: The collected leaves were first washed under running tap water for 30 minutes, followed by surface sterilization with 50% sodium hypochlorite (NaOCl) solution for 20 minutes. Residual bleach was removed by rinsing the leaves four to five times with autoclaved distilled water using an invert mixing technique under a laminar airflow hood (Eeswara *et al.*, 1999; Warisha *et al*., 2019).

Explant culturing: After explant sterilization, each leaf was dissected into four pieces by using a sterile scalpel and aseptically cultured on autoclaved MS media under a laminar airflow hood. The cultured jars were then incubated in darkness at 25-30°C.

Development of neem callus & suspension cultures: The callus and the cell-suspension cultures of *A. indica* were developed by the method of (Warisha *et al*., 2019). The stages of callus induction on MS media from day 1 to day 30 are summarized in Fig. 1 as follows:

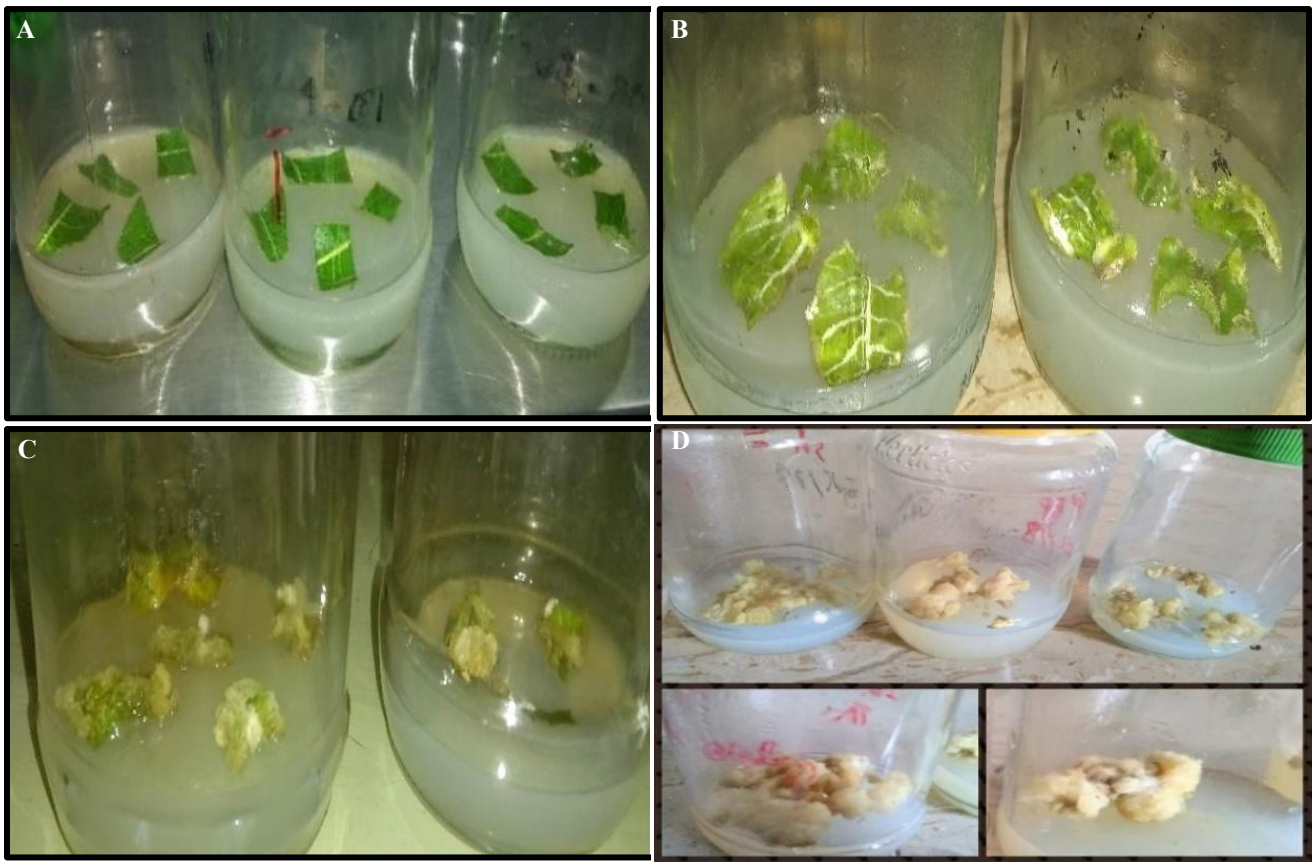

Fig. 1. Culturing of neem leaves on autoclaved solidified MS media **(A).** Within a week, curling and swelling of leaves occurred in most jars and in some, white-colored callus clumps started forming mainly on the midrib and edges of the leaf explants (**B**). The callus size gradually increased by the third week (**C**). Within a month, an off-white friable callus developed and proliferated through sub-culturing on the same fresh medium (**D**).

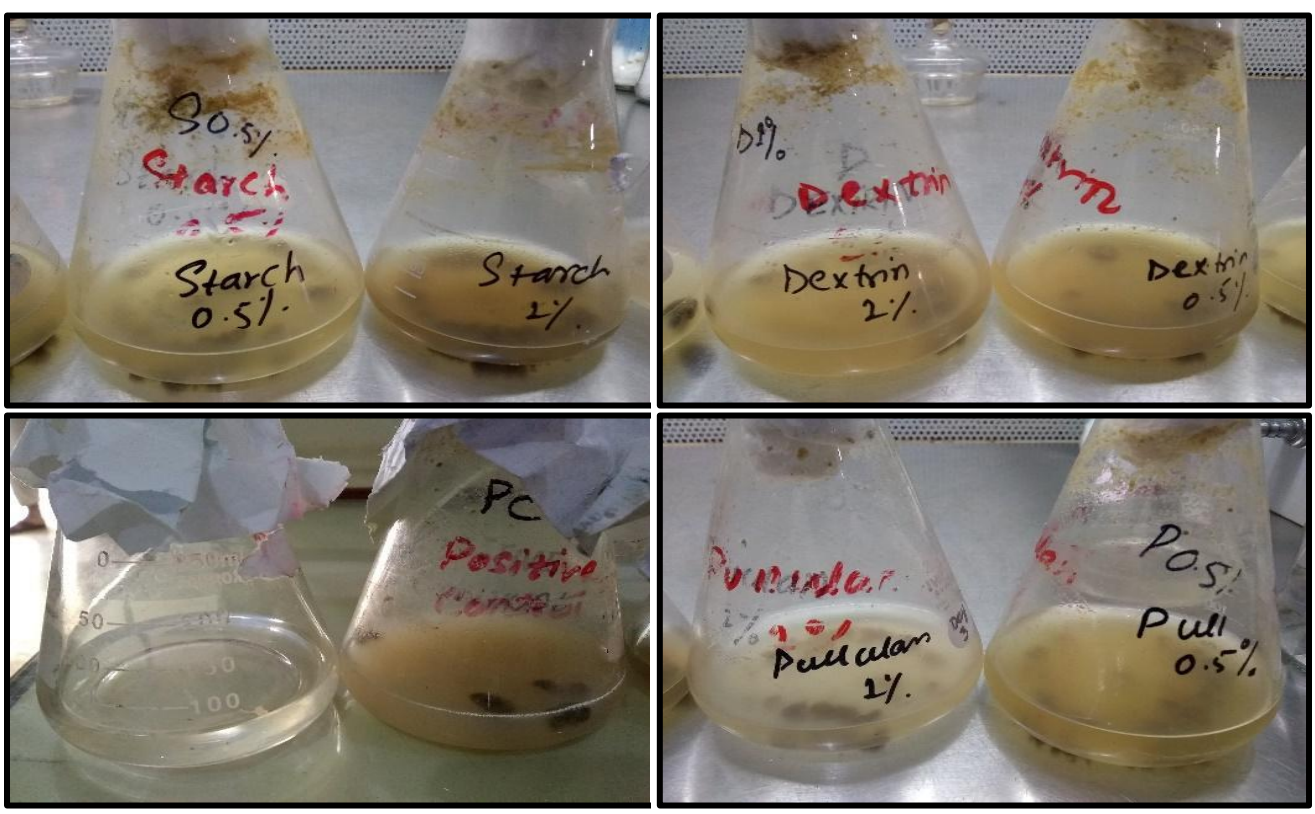

Fig. 2. Neem callus cell suspension cultures were induced with 1 % and 0.5 % of dextrin, starch and pullulan along with positive (PC) and negative control (NC) media. These cultures were continuously agitated on a platform shaker at 25- 30°C and 100 rpm for 16 days.

The cell suspension culture initiation & development: The cell-suspension medium had the same formulation as that of the induction medium, except it lacked agar and BA, also known as 6-benzylaminopurine (BAP). (Fig. 2)

It was supplemented with enzyme inducers namely, starch, dextrin, and pullulan at concentrations of 1% and 0.5%. The pH adjustment and autoclaving conditions for the suspension medium were identical to those of the MS medium. Friable off-white callus clumps were incorporated into the suspension media. The autoclaved medium was labeled as the negative control (NC) while the cultured medium without an inducer served as the positive control (PC).

Cell-free extract preparation: After 16 days of incubation, the suspension medium was separated from the cell mass by filtering it through sterilized Whatman filter paper under a laminar airflow hood. The filtrate, containing extracellular enzymes, was stored at 4°C.

Study of induction effect on enzyme production: The effects of cell-free extracts containing starch, dextrin and pullulan at the concentrations of 0.5% and 1%, as well as positive and negative controls, were analyzed on starch and pullulan agar plates. Four wells were created on each agar plate using a sterilized borer, and 100 ul of extract was added to each well. The incubation time and temperature for the enzymatic reaction on the agar plates are shown in (Table 1).

Preparation of starch and pullulan agar plates: A solution comprising 0.5% starch and 2% agar diluted in distilled water was used to prepare starch agar plates. Before the sterilized medium was transferred into the Petri dishes, the medium and Petri dishes were both autoclaved. Each dish contained approximately 25 ml of the medium. Using 0.5% pullulan and 2% agar, the pullulan agar plates were prepared similarly. The incubation time for enzymatic absorption on agar plates is mentioned in (Table 1). After incubation, the plates were treated with diluted Gram's iodine to analyze hydrolytic zones.

Enzyme assay: The stored filtrate aliquots were thawed in an ice bucket and gradually brought to room temperature. Subsequently, 0.5 ml of the enzyme and substrate solution were mixed and incubated at 37°C for 10 minutes. To terminate the reaction, 1 ml of DNS reagent was added, and the mixture was boiled for 5 minutes. The absorbance of the test solution was measured at 540 nm against a blank, and the amount of maltose released due to enzymatic activity was then calculated using a standard graph.

Estimation of reducing sugars: The estimation of reducing sugars in the reaction mixture was performed using the DNS method (Bernfeld *et al.*, 1955). In this assay, reducing sugars react with 3,5-Dinitrosalicylic acid (DNS), reducing it to 3-amino-5-nitrosalicylic acid abbreviated as ANS, which exhibits strong absorbance at 540 nm. Enzyme activity was quantified based on the standard definition of an enzyme unit where 1 U = 1 µmol of substrate converted per minute (Miller,1959). The micromoles of maltose released were calculated and expressed in enzyme units, as summarized in (Table 2).

Results

Enzyme activity on starch agar plates: The induction effects of starch, dextrin, and pullulan were analyzed using starch agar plates to assess the activities of the amylolytic enzymes. The enzymatic activities varied depending on the inducers used (Figs. 3-5). PC represents the positive control (cultured media without inducer), while NC denotes the negative control (autoclaved MS media). The symbols S, D, and P correspond to starch, dextrin, and pullulan, respectively. CFE refers to the cell-free extract of the induced media.

Starch-induced CFEs: A clear hydrolytic zone was observed for 1% starch-induced CFE, while a slightly clear zone appeared around 0.5% well. No hydrolytic zones were detected around the PC and negative NC wells (Fig. 3). These hydrolytic zones indicate that CFEs of the starch-induced cultured medium resulted in the production of amylolytic enzymes, facilitating starch degradation.

Dextrin-induced CFEs: Clear large hydrolytic zones were observed for both 0.5% and 1% dextrin-induced CFEs. No hydrolytic zones were visible around positive and negative control wells in (Fig. 3). These results indicate that dextrin serves as a strong inducer for amylolytic enzymes, likely including dextrinase and other amylases.

Table 1. Incubation time and temperature for enzyme-substrate reaction on solidified agar plates.

S. No.	Incubation time (hours)	Incubation temperature (◦C)	Substrate- Enzyme
01	24	37	Dextrin- LD, Amylases
02	24	37	Pullulan-LD/Pullulanase, β- amylase
03	24	37	Starch- Amylolytic enzymes

Table 2. The Determination of concentrations of amylolytic enzymes in neem suspension culture by DNS method.

S. No.	Substrates incorporated into suspension media	Absorbances (540 nm)	Maltose concentration from Standard graph (mg)	Maltose concentration from Standard graph (µmol /ml)	Enzyme concentration (mU /ml)
01.	Pullulan (1%)	0.010	0.025 mg	0.138	138
02.	Starch (1%)	0.021	0.045mg	0.248	248
03.	Dextrin (1%)	0.050	0.10 mg	0.554	554
04.	Pullulan (0.5%)	0.029	0.065 mg	0.360	360
05.	Starch (0.5%)	0.027	0.058 mg	0.321	321
06.	Dextrin (0.5%)	0.041	0.085 mg	0.471	471

To calculate enzyme units, the concentration unit of maltose were converted from mg into micromoles (µmol). Using the following formula 1 µmol = ug of substance/ Molecular weight of substance. (Molecular weight of maltose = 360.32 g/mol)

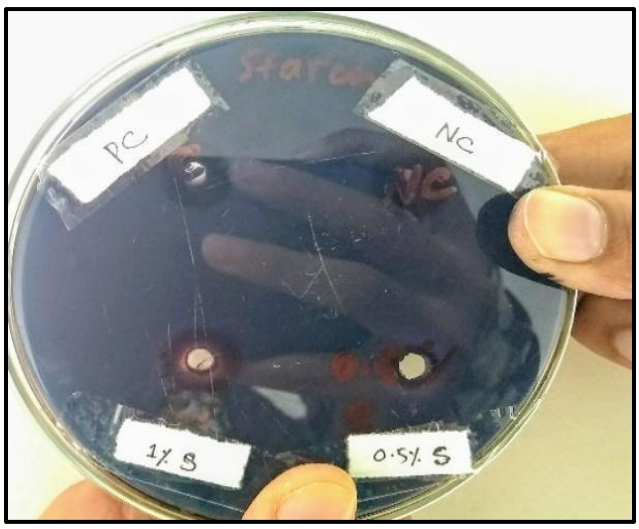

Fig. 3. The activities of amylolytic enzymes resulting from starch-induced CFEs were observed on starch agar plate treated with diluted Gram's iodine.

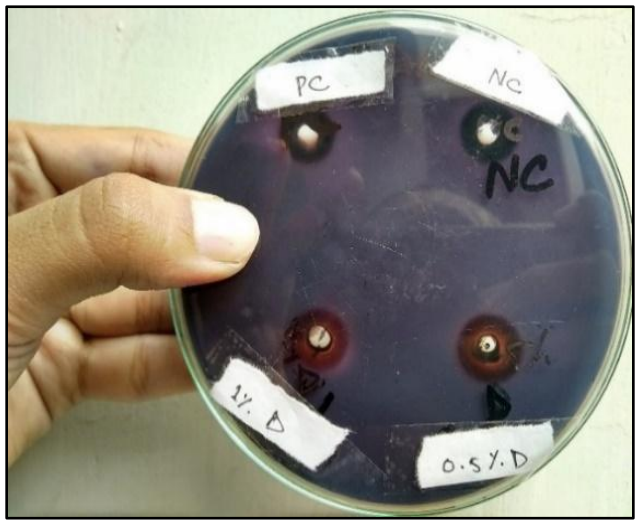

Fig. 4. Activities of dextrinase /amylase resulting from dextrin-induced CFEs were observed on starch agar plate treated with diluted Gram's iodine.

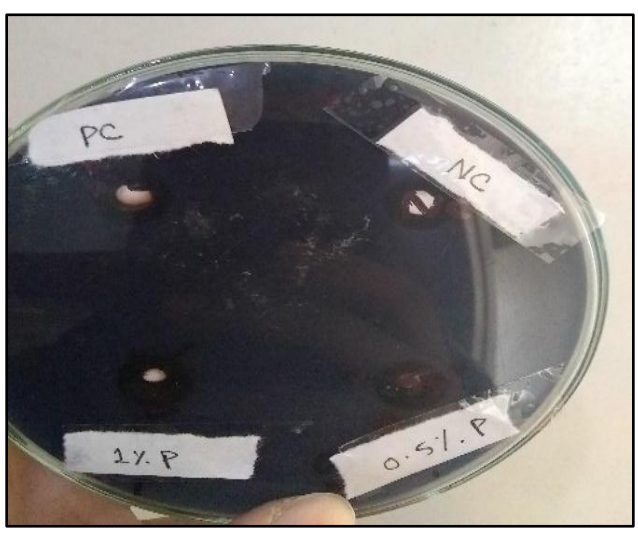

Fig. 5. Pullulan-induced CFEs resulted in no enzymatic activity for each well on the starch agar plate treated with diluted Gram's iodine.

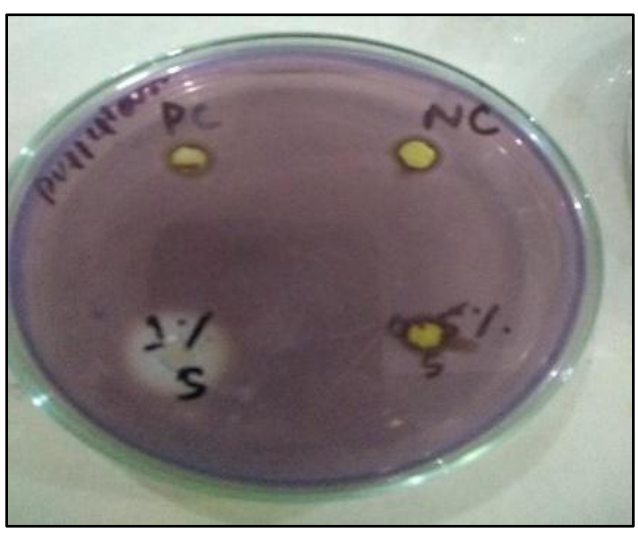

Fig. 6. Activities of amylolytic enzymes observed on pullulan agar plate treated with diluted Gram's iodine.

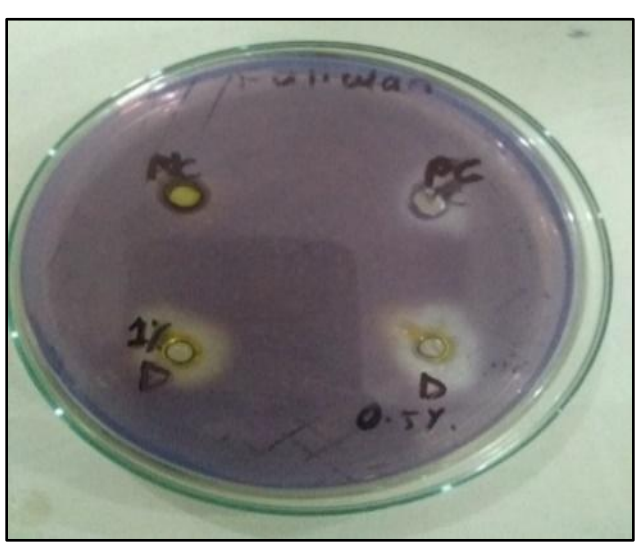

Fig.7. Activities of dextrin-induced CFEs observed on pullulan agar plate treated with diluted Gram's iodine.

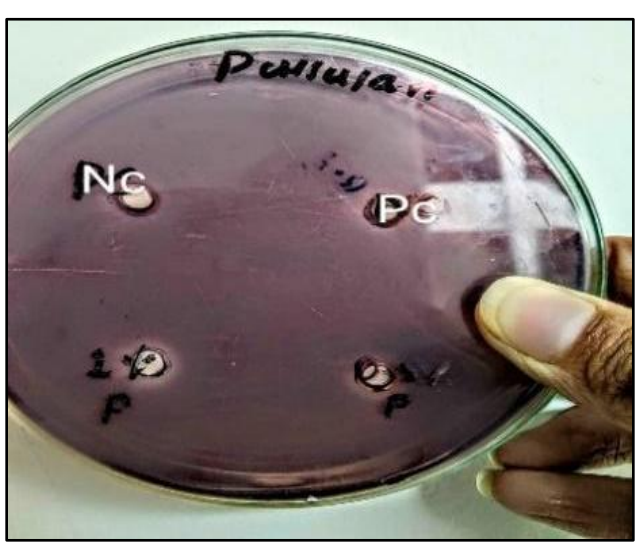

Fig. 8. Activity of pullulanase observed on pullulan agar plate treated with diluted Gram's iodine.

Comparative analysis

Table 3. Comparative qualitative analysis among different substrates in inducing amylolytic and pullulytic enzymes.

S. No.	Effects observed on starch agar plate				
	Substrate	**0.5%**	**1%**	**PC**	**NC**
01	Starch	+/-	++	–	–
02	Dextrin	++	++	–	–
03	Pullulan	–	–	–	–
S. No.	**Effects observed on pullulan agar plate**				
	Substrate	**0.5%**	**1%**	**PC**	**NC**
01	Starch	–	+++	–	–
02	Dextrin	+++	+++	+	–
03	Pullulan	+/-	+	+/-	–

+/-= Very low activity; + = Low activity; ++ = Moderate activity; +++ = High activity; – = No activity

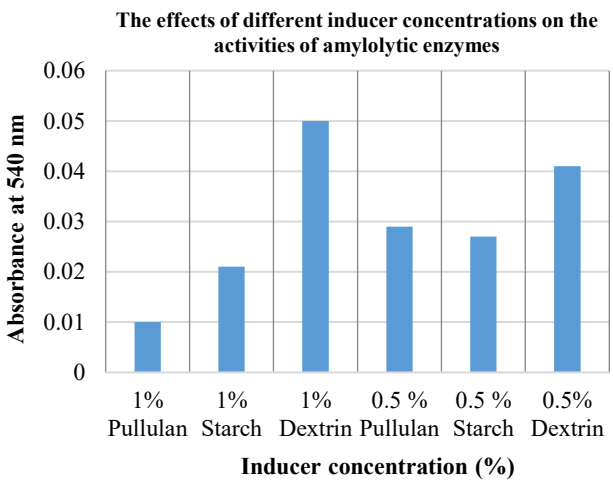

Fig. 9. The effects of varying concentrations of starch, dextrin, and pullulan on the absorbance values resulting from the activities of induced amylolytic & pullulytic enzymes in Neem cell-suspension culture.

Pullulan-induced CFEs: No hydrolytic activity was detected in the wells containing pullulan-induced CFEs as shown in (Fig. 5). This suggested that pullulanase was either not significantly induced or the absence of the synergistic effect of amylases and debranching enzymes hindered starch hydrolysis.

Enzyme activity on pullulan agar plates: To further examine the effects of these inducers on pullulanase and related enzyme activities, pullulan agar plates were used.

Starch-induced CFEs: Significant enzyme activity was detected in 1% pullulan-induced well, whereas no hydrolytic activity was observed around 0.5%, PC, and NC wells as shown in (Fig. 6). These observations indicated pullulan-degrading enzymes such as pullulanase and other related amylases (e.g., β- amylase or glucoamylase), were present in starch-induced extract, leading to pullulan degradation.

Dextrin-induced CFEs: Clear large hydrolytic zones were observed around the wells containing 1% and 0.5% dextrin-induced CFE (Fig. 7). A hydrolytic zone also appeared around the PC, indicating the potential of neem cells to express dextrinase naturally. No hydrolytic zone was observed around the NC.

Pullulan-induced CFEs: Hydrolytic zones were well observed around the wells containing 0.5% and 1% pullulan-induced CFEs, as well as around the PC, (Fig. 8). No zone appeared around the NC well. These hydrolytic activities in CFEs indicate the potential of neem cells to produce pullulanse.

These results indicate that dextrin at both 0.5% and 1% concentrations effectively induced enzyme production in the cell suspension media. The dextrin-induced activities were evident on both starch and pullulan agar plates. Conversely, pullulan-induced CFEs resulted in hydrolytic activity only on pullulan agar plates. Notably, the difference between the results generated by 1% and 0.5% pullulan inducers was minimal. Contrary, the starch-induced CFEs results in qualitative analysis indicate increased enzyme production with higher starch concentration. The enzyme induction potential of substrates in a qualitative test was observed in the order: of Dextrin > Starch > Pullulan**.** A similar trend was noted in quantitative testing with a 1% concentration of starch, dextrin, and pullulan. However, at 0.5% concentration of the same inducers, pullulan's effect was greater than that of starch (Fig. 9).

Discussion

The results demonstrate that neem cells are capable of producing both amylolytic and pullulytic enzymes when induced with their respective inducers in cell suspension media. In both qualitative and quantitative analyses, dextrin proved to be the most effective inducer of amylolytic enzymes. The hydrolytic zones observed on starch agar plates, likely result from the combined activity of multiple amylolytic enzymes with varying specificities for starch breakdown (Martin & Stephan, 2007). Dextrinase alone does not hydrolyze starch granules effectively; instead, a synergistic action between dextrinase, exoamylases, and endoamylases facilitates starch degradation. In contrast, the absence of hydrolytic zones on starch agar plates from pullulan-induced CFEs may be due to either insufficient pullulanase induction or inadequate synergistic action between amylases and debranching enzymes as supported by existing research. The qualitative effects resulting from different percentages of starch contradict quantitative results, however, it has been previously stated that "high starch concentration down-regulates amylase expression in callus culture" (warisha *et al.*, 2019) and hence satisfies quantitative analysis.

Pullulan serves as the primary substrate for pullulanase, yet the enzyme also hydrolyzes α-(1-6)-linked maltose in amylopectin and β-limit dextrins (Van Der Maarel *et al*., 2002). The hydrolytic activities observed on pullulan agar plates from starch, dextrin, and pullulan-induced CFEs indicate the successful induction of pullulytic enzymes. Additionally, the hydrolytic zones observed on pullulan agar plates from dextrin-induced CFEs further confirm the production of pullulytic enzymes. Slow pullulan degradation by α-amylase, glucoamylase, and rapid lysis by β- amylase is also reported by (Bruneel & Schacht, 1995). Hence, the hydrolytic zones observed on pullulan agar plates from pullulan-induced CFE are likely due to the activity of pullulanase which effectively hydrolyzes α-1,6 glycosidic bonds in pullulan rather than β- amylase which primarily acts on α-1,4 linkages in starch and has limited activity towards pullulan (Beck & Ziegler, 1989; Adachi *et al*., 1998). The absence of hydrolytic zones for positive control well on the solidified plates may be due to the thickness of agar in the plate or interference by secondary metabolites such as azadirachtin, tannins, saponins, and flavonoids in the plant extract (Kazeem *et al*., 2013; Sami *et al*., 2014). Further investigation including, purification, and functional characterization of these enzymes will provide more insights into the synergistic interactions influencing their substrate specificity and activity.

Conclusion

Consequently, this research demonstrates the ability of neem-cultured cells to induce both amylolytic and pullulytic enzymes. However, the potential of neem for amylases appears to be greater than that for pullulanase, as hydrolytic activities were stimulated mostly by starch and dextrin.

Partially purifying the cell suspension culture may further enhance enzyme production by reducing interference from the secondary metabolites. Additionally, optimizing the inducer concentration could further improve enzyme yield. Lastly, purifying and characterizing these enzymes will offer deeper insights into this study, paving the way for future researchers to utilize this natural, non-pathogenic plant source for commercial-scale enzyme production.

References

Aoki, H. and Y. Sakano. 1997. A classification of dextran-hydrolysing enzymes based on amino-acid-sequence similarities. *Biochem. J.*, 323(3): 859-861.

Adachi, M., B. Mikami, T. Katsube and S. Utsumi. 1998. Crystal structure of recombinant soybean beta-amylase complexed with beta-cyclodextrin. *J. Biol. Chem.*, 273(31): 19859-19865. https://doi.org/10.1074/jbc.273.31.19859

Alsanosi, S.M.M., C. Skiffington and S. Padmanabhan. 2014. Pharmacokinetics pharmacogenomics. In: (Ed.): Padmanabhan, S. *Handbook of Pharmacogenomics and Stratified Medicine.* Academic Press, London, pp. 341-364. http://dx.doi.org/10.1016/B978-0-12-386882-4.00017-7

Ball, S.G., H.P. Guan, M.G. James, A.M. Myers, P. Keeling, G. Mouille, A. Buléon, P. Colonna and J. Preiss. 1996. From glycogen to amylopectin: A model for the biogenesis of the plant starch granule. *Cell*, 86(3): 349-352. https://doi.org/10.1016/S0092-8674(00)80107-5

Beatty, M.K., A. Rahman, H. Cao, W. Woodman, M. Lee, A.M. Myer and M.G. James. 1999. Purification and molecular genetic characterization of ZPU1, a pullulanase-type starch debranching enzyme from maize. *Plant Physiol*., 119(1): 255-266.

Beck, E. and P. Ziegler. 1989. Biosynthesis and degradation of starch in higher plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 40: 95-117. https://doi.org/10.1146/annurev.pp.40.060189.000523.

Bernfeld, P. 1955. Amylases, α and β. *Methods Enzymol.,* 1: 149-158.

Brahmachari, G. 2004. Neem-an Omnipotent Plant*: a Retrospection. Chem Bio-Chem.,* 5(4): 408-421.

Bruneel., D. and E. Schacht. 1995. Enzymatic degradation of pullulan and pullulan derivatives. *J. Bioact. Compact. Polym.*, 10(4): 299-312. https://doi.org/10.1177/088391159501000403.

Buléon, A., P. Colonna, V. Planchot and S. Ball. 1998. Starch granules: structure and biosynthesis. *Int. J. Biol. Macromol*., 23(2): 85-112.https://doi.org/10.1016/s0141-8130(98)00040-3.

Cho, M.J., J.H. Wong, C. Marx, W. Jiang, P.G. Lemaux and B.B. Buchanan. 1999. Overexpression of thioredoxin h leads to enhanced activity of starch debranching enzyme (pullulanase) in barley grain. *Proc. Natl. Acad. Sci. U.S.A*., 96(25): 14641-14646. https://doi.org/10.1073/pnas.96.25.14641

Dinges, J.R., C. Colleoni, M.G. James and A.M. Myers. 2003. Mutational analysis of the pullulanase-type debranching enzyme of maize indicates multiple functions in starch metabolism. *Plant Cell*, 15(3): 666-680.

Drummond, G.S. 1970. Action of polysaccharide branching and debranching enzymes. University of Miami.

Dunn, G., D.G. Hardie and D.J. Manners. 1973. Observations on the action of limit dextrinases on amylopectin-like polysaccharides. *Biochem. J*., 133(2): 413.

Eeswara, J.P., E.J. Allan, J. Mordue and T. Stuchbury. 1999. A procedure for sterilizing leaf explants collected from wild neem (*Azadirachta indica*) trees. *J. Natl. Sci. Found. Sri Lanka*., 27(2): 131-136. doi: 10.4038/jnsfsr.v27i2.2986.

El-Shora, H.M., S.E. Ghada and A.H.S. Amany. 2015. Purification, thermostability, and chemical modification of pullulanase from sunflower. *Int. J. Curr. Microbiol. Appl. Sci*., 4(12): 284-296.

Fogarty, W.M. 1983. Microbial amylases. In: (Eds.): Fogarty, W.M. and C.T. Kelly. *Microbial Enzymes and Biotechnology.* Applied Science Publishers. pp. 1-92.

Francisco, P.B. Jr., Y. Zhang, S.Y. Park, H. Ogata and Y. Nakamura. 1998. The Genomic DNA sequence of a rice gene coding for a pullulanase-type of starch debranching enzyme. *Biochim. Biophys. Acta*, 1387(1-2): 469-477. https://doi.org/10.1016/s0167-4838(98)00154-x.

Gordon, R.W., D.J. Manners and J.R. Stark. 1975. The limit dextrinase of the broad bean (*Vicia faba* L.). *Carbohydr. Res*., 42(1): 125-134.

Gupta, A.K. and S.R. Chaphalkar. 2015. Analytical studies of protease extracted from *Azadirachta indica*. *World J. Pharm. Res*., 4(11): 1391-1398.

Guzmán-Maldonado, H. and O. Paredes-López. 1995. Amylolytic enzymes and products derived from starch: a review. *Crit. Rev. Food Sci. Nutr*., 35(5): 373-403. https://doi.org/10.1080/10408399509527706

Haki, G.D. and S.K. Rakshit. 2003. Developments in industrially important thermostable enzymes: A review. *Bioresour. Technol.*, 89(1): 17-34. https://doi.org/10.1016/s0960-8524(03)00033-6

Hardie, D.G., D.J. Manners and D. Yellowlees. 1976. The limit dextrinase from malted sorghum (*Sorghum vulgare*). *Carbohydr. Res*., 50(1): 75-85.

Hii, S.L., J.S. Tan, T.C. Ling and A.B. Ariff. 2012. Pullulanase: Role in starch hydrolysis and potential industrial applications. *Enzyme Res.*, 921-362. https://doi.org/10.1155/2012/921362.

James, M.G., K. Denyer and A.M. Myers. 2003. Starch synthesis in the cereal endosperm. *Curr. Opin. Plant Biol.*, 6(3): 215-222.

Janecek, S. 2009. Amylolytic enzymes–focus on the alpha-amylases from Archaea and plants. *Nova Biotechnol*., 9. doi:10.36547/nbc.1284.

Kazeem, M.I., T.V. Dansu and S.A. Adeola. 2013. Inhibitory effect of *Azadirachta indica* A. Juss leaf extract on the activities of α-amylase and α-glucosidase. *Pak. J. Biol. Sci.*, 16(21): 1358-1362.

Kearney, M.L., E.J. Allan, J.E. Hooker and A.J. Mordue. 1994. Antifeedant effects of in vitro culture extracts of the neem tree, *Azadirachta indica*, against the desert locust (*Schistocerca gregaria* (Forskål)). *Plant Cell Tissue Organ Cult*., 37: 67-71.

Kedderis, G.L. 2010. Biotransformation of Toxicants. In: *Comprehensive Toxicology*. pp. 137-151. doi:10.1016/B978-0-08-046884-6.00107-X.

Koul, O. and S. Wahab. (Eds.). 2004. Neem: today and in the new millennium. Kluwer Academic Publishers, Dordrecht, pp. 276.

Kubo, A., N. Fujita, K. Harada, T. Matsuda, H. Satoh and Y. Nakamura. 1999. The starch-debranching enzymes isoamylase and pullulanase are both involved in amylopectin biosynthesis in rice endosperm. *Plant Physiol.*, 121(2): 399-410.

Lee, E.Y.C. and W.J. Whelan. 1971. Glycogen and starch debranching enzymes. In: (Ed.): Boyer, P.D. The Enzymes, Vol: 5. Academic Press. pp. 191-234.

Li, B., J.C. Servaites and D.R. Geiger. 1992. Characterization and subcellular localization of debranching enzyme and endoamylase from leaves of sugar beet. *Plant Physiol.*, 98(4): 1277-1284.

Manjula, N., P. Suneetha, K.N. Lokesh, M. Prabha, J.A. Bosse, G.N. Makarla and M. Kushalatha. 2017. Production of lipase from *Azadirachta indica* oil seed cake using solid-state fermentation. *Int. J. Appl. Res.*, 3(9): 223-230.

Martin, M. and J. Štefan. 2007. Amylolytic enzymes: types, structures, and specificities. In: (Eds.): Polaina, J. and A.P. MacCabe. *Industrial Enzymes: Structure, Function and Applications*. Springer Netherlands, Dordrecht, pp. 3-18.

Miller, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.*, 31(3): 426-428.

Morinaga, T., E. Nakano and S. Fukuda. 1997. Purification and characterization of a novel pullulanase from *Thermus aquaticus* ATCC 33923. *J. Ferment. Bioeng.*, 83(5): 461-465. doi: 10.1271/bbb.60.835

Mouille, G., M.L. Maddelein, N. Libessart, P. Talaga, A. Decq, B. Delrue and S. Ball. 1996. Preamylopectin processing: A mandatory step for starch biosynthesis in plants. *Plant Cell*, 8(8): 1353-1366.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*., 15(3): 473-497.

Naik, B., V. Kumar, S.K. Goyal, A. Dutt Tripathi, S. Mishra, P.E. Joakim Saris and S. Rustagi. 2023. Pullulanase: Unleashing the power of enzymes with a promising future in the food industry. *Front. Bioeng. Biotechnol*., 11: 1139611.

Nakamura, Y., A. Kubo, T. Shimamune, T. Matsuda, K. Harada and H. Satoh. 1997. Correlation between activities of starch debranching enzyme and α-polyglucan structure in endosperms of sugary-1 mutants of rice. *Plant J.*, 12(1): 143-153.

Anonymous. 1992. Neem: A tree for solving global problems, report of an ad-hoc panel of the Board on Science and Technology for International Development. National Academy Press, Washington, DC.

Netrphan, S. 2002. Study of starch debranching enzymes in developing wheat kernels. Doctoral dissertation, University of Saskatchewan.

Nicoletti, M. and K. Murugan. 2013. Neem: The tree of the 21st century. *Pharmacologyonline*, 3: 115-121.

Okita, T.W. and J. Preiss. 1980. Starch degradation in spinach leaves: isolation and characterization of the amylases and R-enzyme of spinach leaves. *Plant Physiol*., 66(5): 870-876.

Pan, D. and O.E. Nelson. 1984. A debranching enzyme deficiency in endosperms of the sugary-1 mutants of maize. *Plant Physiol.*, 74(2): 324-328.

Pandey, A., P. Nigam, C.R. Soccol, V.T. Soccol, D. Singh and R. Mohan. 2000. Advances in microbial amylases. *Biotechnol. Appl. Biochem.*, 31(2): 135-152. https://doi.org/10.1042/ba19990073

Peng, Y.L. and F. Hu. 2012. Purification and characterization of limit dextrinase from malted barley. *Adv. Mater. Res.*, 550: 1747-1754.

Sakano, Y., N. Masuda and T. Kobayashi. 1971. Hydrolysis of pullulan by a novel enzyme from *Aspergillus niger*. *Agric. Biol. Chem*., 35(6): 971-973. https://doi.org/10.1271/bbb1961.35.97

Sakano, Y., N. Masuda and T. Kobayashi. 1971. Hydrolysis of pullulan by a novel enzyme from Aspergillus niger. *Agric. Biol. Chem*., 35(6): 971-973. https://doi.org/10.1271/bbb1961.35.97

Saleem, S., G. Muhammad, M.A. Hussain and S.N.A. Bukhari. 2018. A comprehensive review of phytochemical profile, bioactives for pharmaceuticals, and pharmacological attributes of *Azadirachta indica*. *Phytother. Res*., 32(7): 1241-1272. https://doi.org/10.1002/ptr.6076

Sami, A.J. 2014. *Azadirachta indica*-derived compounds as inhibitors of digestive α-amylase in insect pests: Potential bio-pesticides in insect pest management. *Eur. J. Exp. Biol.*, 4(1): 259-264.

Vaidya, S., P.K. Srivastava, P. Rathore and A.K. Pandey. 2015. Amylases: A prospective enzyme in the field of biotechnology. *J. Appl. Biosci.*, 41(1): 1-18.

Van Der Maarel, M.J., B. Van der Veen, J.C. Uitdehaag, H. Leemhuis and L. Dijkhuizen. 2002. Properties and applications of starch-converting enzymes of the α-amylase family. *J. Biotechnol*., 94(2): 137-155.

Verified Market Research. 2024. Global pullulanase market size by application, by end-user industry, by Geographic Scope and Forecast. https://www.verifiedmarketresearch.com

Vihinen, M. and P. Mantsiila. 1989. Microbial amylolytic enzyme. *Crit. Rev. Biochem. Mol. Biol.*, 24(4): 329-418.

Wangpaiboon, K., T. Charoenwongpaiboon, M. Klaewkla, R.A. Field and P. Panpetch. 2023. Cassava pullulanase and its synergistic debranching action with isoamylase 3 in starch catabolism. *Front. Plant Sci*., 14: 1114215.

Warisha, A., A. Awais, S.A. Hassan and Saifullah. 2019. Induction of the dextrinase and other hydrolysing enzymes in the suspension culture of *Azadirachta indica* A. Juss. *Int. J. Biol. Biotech*., 16 (2): 267-276.

Wu, C., C. Colleoni, A.M. Myers and M.G. James. 2002. Enzymatic properties and regulation of ZPU1, the maize pullulanase-type starch debranching enzyme. *Arch. Biochem. Biophys.*, 406(1): 21-32.

Yamada, J. and M. Izawa. 1979. Mode of action of a rice debranching enzyme on pullulan and its related oligosaccharides. *Agric. Biol. Chem*., 43(12): 2515-2521.

Yamada, J. 1981. Purification of oat debranching enzyme and occurrence of inactive debranching enzyme in cereals. *Agric. Biol. Chem*., 45(4): 1013-1015.

Yamasaki, Y., S. Nakashima and H. Konno. 2008. Pullulanase from rice endosperm. *Acta Biochim. Pol*., 55(3): 507-510.

Zhu, Z.P., C.M. Hylton, U. Rößner and A.M. Smith. 1998. Characterization of starch-debranching enzymes in pea embryos. *Plant Physiol.*, 118(2): 581-590. https://doi.org/10.1104/pp.118.2.581.

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