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Production and purification of extracellular pullulanase by *Klebsilla aerogenes* NCIM 2239

N. Prabhu[1], R. Uma maheswari[1], M. Vijay Pradhap Singh[1], S. Karunakaran[1], C. Kaliappan[2] and T. Gajendran[2*]

[1]Department of Biotechnology, Vivekanandha College of Engineering for Women, Elayampalayam, Namakkal-637 205, Tamilnadu, India.
[2]Department of Biotechnology, Bharathidasan Institute of Technology, Anna University, Tiruchirappalli-620 024, Tamilnadu, India.

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The present study was carried out to optimize the production of pullulanase from *Klebsilla aerogenes* NCIM 2239, a new strain of the bacterium. The organism was screened for its ability to synthesize and secrete extracellular pullulanase, by analysis of pullulan degrading activity. Environmental (pH and temperature) and nutritional (carbon and nitrogen) factors influencing growth and product formation were optimized through fermentation trials in shake flasks. Maximum pullulanase production (78.62 U/ml) was observed at 48 h. Optimized pH and temperature were 7.0 and 37°C, respectively. Different carbon sources (pullulan, starch, sucrose, maltose and glucose) and nitrogen sources (peptone, beef extract, yeast extract and casein) were analysed for maximum production of pullulanase. This bacterium was found to exhibit maximum pullulanase production using starch and peptone as carbon and nitrogen sources, respectively. In addition, pullulanase production by solid substrate fermentation (SSF) was investigated using *K. aerogenes* NCIM 2239. The extracellular pullulanase was purified by ultrafiltration method of membrane separation. The pullulanase activity after ultrafiltration was 130.21 U/ml when compared with its crude (83.08 U/ml).

Key words: Pullulanase, *α*-1,6 glucosidic linkages, *Klebsilla aerogenes* NCIM 2239, solid state fermentation, ultrafiltration.

INTRODUCTION

Pullulanase (pullulan α-glucanohydrolase (EC 3.2.1.41) is an extracellular carbohydrase which was first discovered by Bender and Wallenfels in 1961 from mesophilic organism, *Klebsiella pneumoniae* (Murooka et al., 1989). Pullulanases are also called de-branching enzymes and have been widely used to hydrolyse the *α*-1, 6 glycosidic linkages in starch, amylopectin, pullulan and related oligosaccharides (Hii et al., 2012; Duan and Wu, 2015) (Figure 1).

Pullulan, a linear α-glucan was synthesised by *Pullularia pullulan* (Bender and Wallenfels, 1961; Catley, 1971). It consists of repeating units of α-maltotriose

*Corresponding author. E-mail:gajendran01@yahoo.co.in.

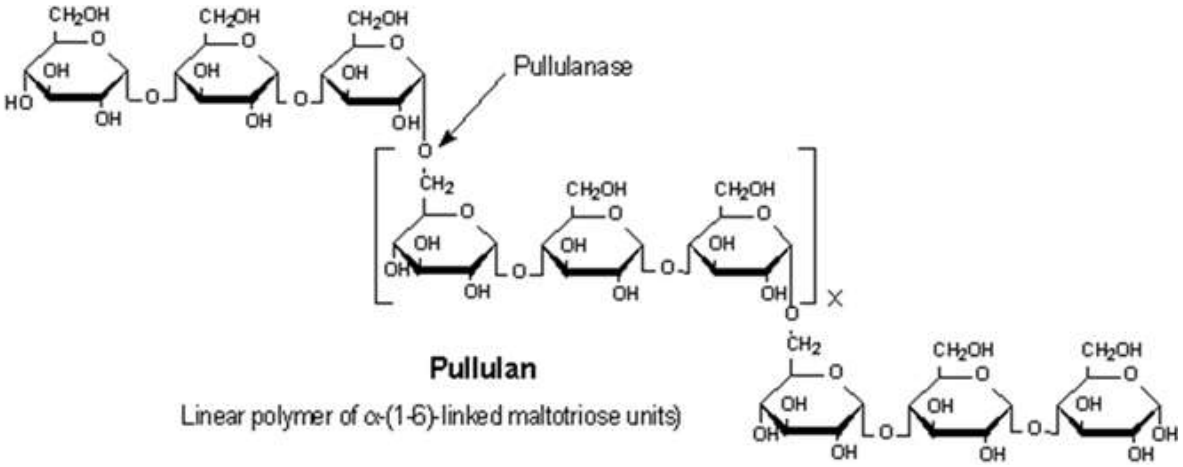

Figure 1. Pullulan degradation by pullulanase (Hii et al., 2012).

joined “head to tail” by α-1, 6 bonds. Pullulanase specifically attacks α-1, 6-glycosidic linkage of branched chains as well as α-1, 6-glycosidic and α-1, 4-glycosidic linkages within other polysaccharide residues; these properties have made pullulanase, a useful agent in structural studies of oligosaccharide and polysaccharide (Drummond et al., 1969).

A good number of pullulanases has been purified and characterized from different bacterial sources. Pullulanase type I has been characterized from mesophilic bacteria such as *Aerobacter aerogenes* (Ohba et al., 1973), *Bacillus acidopullulyticus* (Jensen et al., 1984), *K. pneumonia* (Bender and Wallenfels, 1961; Kornacker et al., 1990) and *Streptomyces* sp. (Takasaki et al., 1993). Moderate thermophilic gram positive bacteria such as *Bacillus flavocaldarius* (Suzuki et al., 1991), *Bacillus thermoleovorans* (Ben Messaoud et al., 2002), *Clostridium* sp. (Klingeberg et al., 1990) and *Thermos caldophilus* (Kim et al., 1996) also have the ability to secrete pullulanase type I, while pullulanase type I from hyperthermophilic bacterium, *Fervidobacterium pennavorans,* has also been reported (Koch et al., 1997). Unlike pullulanase type I, pullulanase type II is widely distributed among extreme thermophilic bacteria and Hyperthermophilic *archaea* (Sunna et al., 1997). The most thermostable and thermoactive pullulanase type II reported to date, was derived from hyperthermophilic archaeon, *Pyrococcus woesei* (Rudiger et al., 1995) and *Pyrococcus furiosus* (Brown and Kelly, 1993; Brown et al., 1990). The current study deals with the production of pullulanase from *Klebsilla aerogenes* NCIM 2239.

Pullulanase is of great significance due to its wide area of potential application. Pullulanase is widely used in industries in the saccharification of starch (Prakash et al., 2012). It converts starch into glucose and maltose which are used in the production of glucose syrup more efficiently. The products obtained by the action of pullulanase have various pharmaceutical and neutraceutical properties. Pullulanase is inexpensive and easily available hence, it helps in the economical production of various products like resistant starch and substitutes of locust bean gum (LBG). The saccharification property of this enzyme has led to its use in various food industries for the production of glucose syrup, maltose syrup and beer (Shaw et al., 1992). Bioethanol production could also be done by the use of pullulanase thus providing a source of fuel. The basic property of breaking α linkage has been widely used by the baking industry (Prakash et al., 2012).

The aim of this research work was to optimize the parameters of fermentation such as carbon sources, nitrogen sources, pH, temperature and incubation time for the enhancement of production of pullulanase from *K. aerogenes* NCIM 2239 and to purify the enzyme by ultrafiltration method. In addition, the effect of submerged fermentation and solid state fermentation was studied.

MATERIALS AND METHODS

Microorganism

The pullulanase-producing bacterium, *K. aerogenes* NCIM 2239, was procured from National Collection of Industrial Microorganisms (NCIM) and National Chemical Laboratory at Pune, India.

Inoculum preparation

The strains were maintained by subculturing consecutively in maltose limiting medium at 37°C under aerobic conditions. For inoculum preparation, a loop from the stock was sub-cultured into the nutrient broth.

Single colony from the nutrient agar was inoculated into nutrient broth and incubated to obtain initial cell concentration. This culture was used as inoculum (1% v/v) for all fermentations carried out in this process.

Production of pullulanase

Extracellular pullulanase secretion by *K. aerogenes* NCIM 2239 was obtained in a modified mineral Czapek medium (Hii et al., 2009) with maltose as a carbon source. The culture medium consisted of (w/v): K_2HPO_4 (0.1%), $NaNO_3$ (0.5%), $MgSO_4.7H_2O$ (0.05%), KCl (0.05%), $FeSO_4.7H_2O$ (0.001%), peptone (0.8%) and maltose (0.5%).

The pH of all media was adjusted to 7.0 and sterilized at 121°C for 20 min. After incubation of the cultures at 37°C for 2 days, the cells were removed from the culture medium by centrifugation at 10,000 rpm for 10 min.

Determination of pullulanase activity

Enzyme activity was determined by measuring the enzymatic release of reducing sugar from pullulan. In this assay, 0.5 mL of enzyme sample was added into 0.5 mL of 1% (w/v) pullulan in 0.02 M sodium phosphate buffer at pH 7.0. The reaction mixture was incubated at 40°C for 30 min and the amount of reducing sugar released at the end of the reaction was determined by dinitrosalicylic acid (DNSA) method.

Sample blank was used to correct the non-enzymatic release of reducing sugar. One unit of pullulanase activity is defined as the amount of enzyme required to produce 1 µ mol reducing sugar (equivalent to glucose) per minutes under the assay conditions (Ling et al., 2009).

Optimum conditions for pullulanase production

Effect of incubation period

A volume of 100 ml of selected medium was taken in each 250 ml conical flask. All flasks were autoclaved at 121°C at 20 min. After cooling, the flasks were inoculated. The flasks were incubated for 1 to 3 days. The cells were precipitated by centrifugation at 10000 rpm. From the supernatant, enzyme activity was measured.

Effect of incubation temperature

The culture medium was incubated at different temperature for optimum enzyme production. For this reason, equal quantity of inoculum was added in each conical flask containing 100 ml of selected suitable medium with selected pH.
The flasks were then incubated at different temperature (28, 37, 45 and 55°C), respectively, for optimum enzyme production. The effect of temperature on pullulanase production was recorded.

Effect of pH

To observe the effect of medium pH on enzyme production, 100 ml of selected medium at different pH (5, 6, 7, 8, 9 and 10, respectively) were used. The flasks were then inoculated and incubated in optimum incubation periods. The effect of medium pH on pullulanase activity was recorded.

Effect of carbon sources

The effect of carbon sources on pullulanase production was tested by using different carbon sources namely, sucrose, starch, pullulan, glucose and maltose. They were tested individually at the concentration of 1% in the carbon source-optimized medium.

Effect of nitrogen sources

Nitrogen source is one of the most common factors which affect the pullulanase enzyme production. The organic nitrogen source used greatly influenced both growth and pullulanase production.
The growth medium was initially supplemented with different organic nitrogen sources (0.5%), that is, beef extract, peptone, yeast extract and casein. Then, the nitrogen source that gives maximum enzyme production was optimized.

Solid state fermentation

10 g of rice bran, corn bran, rice bran + corn bran was taken and then, K_2HPO_4 0.1 g, $NaNO_3$ 0.5 g, $MgSO_4.7H_2O$ 0.05 g, KCl 0.05 g, $FeSO_4.7H_2O$ 0.001 g and peptone 0.5 g were autoclaved and inoculated with 2 ml of 24 h activated bacterial suspension, incubated at 37°C for 48 h.

The enzyme was extracted by using 25 ml of distilled water to the solid substrate culture and well mixed by agitation for 5 min, then filtered through a Whatmann paper. The filtrate was centrifuged at 8000 rpm for 20 min. The supernatant was used as crude enzyme and the enzyme activity was assayed.

Purification of pullulanase by ultrafiltration

The enzyme was purified by ultrafiltration method. Since the molecular weight of pullulanase was 98 kDa approximately (Malakar et al., 2012; Shehata et al., 2016), the polyether sulphone membrane of molecular weight cut-off 100 kDa was used.

In this method, the polyether sulphone membrane was fitted in membrane support. The experimental setup was placed above the magnetic stirring plates. In the feed reservoir, 150 ml of crude enzyme was loaded through the peristaltic pump and the pressure was passed for stirring.
Simultaneously, permeate was collected through an outlet (Liu et al., 2009). The collected volume of permeate was analyzed using DNSA method.

RESULTS AND DISCUSSION

Optimum conditions for pullulanase production

Effect of incubation period

The growth of microorganism and their enzymatic activities were estimated during incubation period which extended up to 48 h. The results are shown in Figure 2. The biosynthesis of extra cellular enzyme increased almost linearly until the stationary phase of growth was attained. Growth as well as pullulanase activity reached maximum value after 48 h of incubation.

The effect of incubation period was checked at an interval of 12 h till 72 h. The maximum enzyme activity was observed at 48 h after which the enzyme activity started to decrease. Similar result was reported from pullulanase enzyme from *Bacillus cereus* (Waleed et al., 2015).

The reason for this is the denaturation of the enzyme caused by the interaction with other components in the medium (Lonsane et al., 1990). It could have also been due to the fact that, the microorganism was on its decline

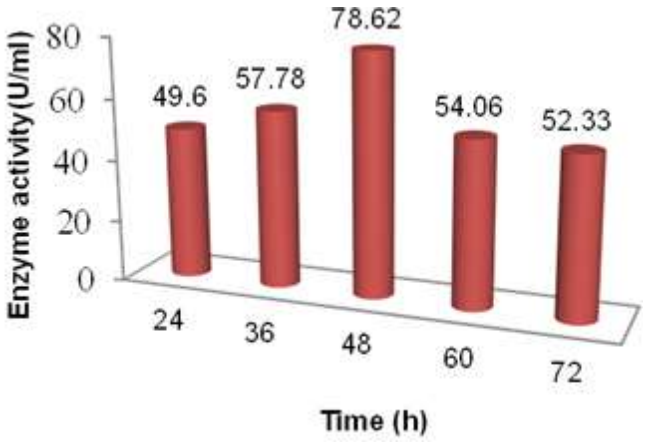

Figure 2. Effect of incubation time on pullulanase production.

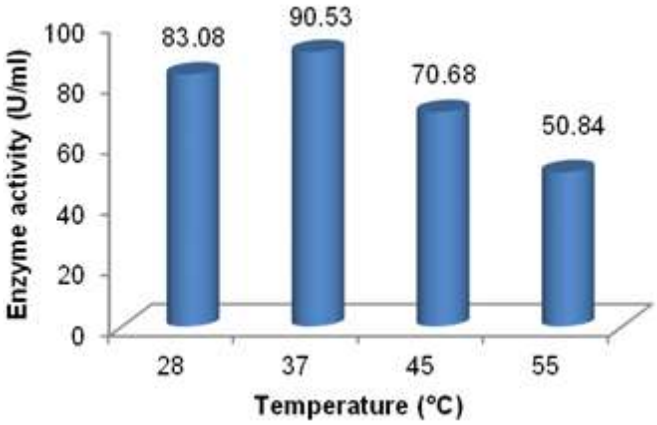

Figure 3. Effect of incubation temperature.

phase during the third day of fermentation which resulted in the decreased enzyme production (Figure 2). During long time exposure, proteins component would denature in the submerged fermentation medium.

Effect of temperature

Temperature is one of the most important parameter governing the growth of organism and enzyme biosynthesis. The enzyme production pattern shows a gradual increase in the enzymatic activity giving maximum production at 37°C. Similar result was reported from pullulanase enzyme from *B. cereus* (Waleed et al., 2015).

At higher temperature due to the production of the large amount of the metabolic heat, the fermenting substrate temperature shoots up, thereby inhibiting microbial growth and enzyme production. The effect of temperature was observed in the range of 28, 37, 45 and 55°C. The maximum enzyme activity was observed at 37°C after which, the enzyme loses its stability and activity decreases (Figure 3).

Similar results were reported for *Aspergillus niger* at 35°C (Lonsane et al., 1985), and for amylase production by *Bacillus* sp. at 37°C, others showed a high level of enzyme production from *Raoultella* which was 30°C (Hii et al., 2009). The optimum condition for amylopullulanase production by *Clostridium thermosulfogenes* was observed at 60°C (Swamy and Seenayya, 1996).

Effect of pH

Microorganisms were grown at different pH in the range of 5 to 10, to determine the effect on pullulanase production. The degree of enzyme production was highest at pH 7and reduces with the increase in pH as described in Figure 4. Similar result was reported from pullulanase enzyme from *B. cereus* (Waleed et al., 2015).

pH can influence the growth and product formation due to its effect on the solubility of nutrient, ionization of the substrate and its availability to the microorganism (Kotwal et al., 1998). Also, the acidic or basic media may lead to denaturation of the enzyme (Bertoldo et al., 2002).

An attempt to overcome the problem of pH variability during the solid substrate fermentation (SSF) process was obtained by the substrate formulation, considering the buffering capacity of the different components employed by the use of buffer formulation with components that have no deleterious influence on the biological activity (Kotwal et al., 1998).

Effect of carbon sources

The effect of different carbon sources on the production of pullulanase was studied using the basal culture medium, supplemented with 1% of carbon sources. The culture containing 1% of each carbon source at a time was incubated and tested for enzymatic activity.

Among these glucose, maltose, pullulan, sucrose and starch were used as 1% at a time. Starch enhanced the pullulanase activity (92.76 U/ml) when compared with other carbon sources. Carbon source is the most effective factor for the production of pullulanase from microorganism.

In the presence of soluble starch, the production of pullulanase was increased as compared to other carbon sources. Maximum production of pullulanase was observed with soluble starch and hence, it is a good source of carbon used in production of pullulanase (Malviya et al., 2010).

The highest pullulanase activity may be due to inducible nature of maltose, being hydrolytic product of starch. Various carbon sources were checked as media component and pullulan was observed with best enzyme

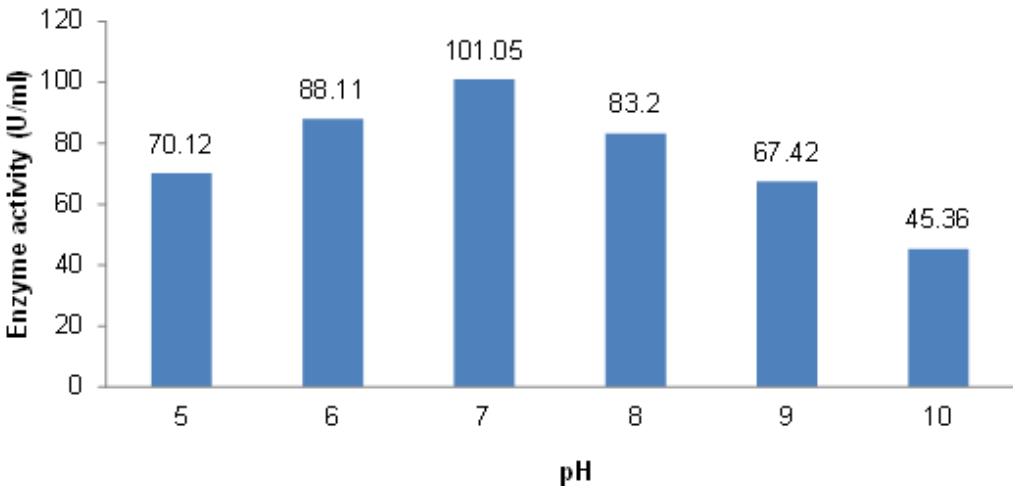

Figure 4. Effect of pH on pullulanase production.

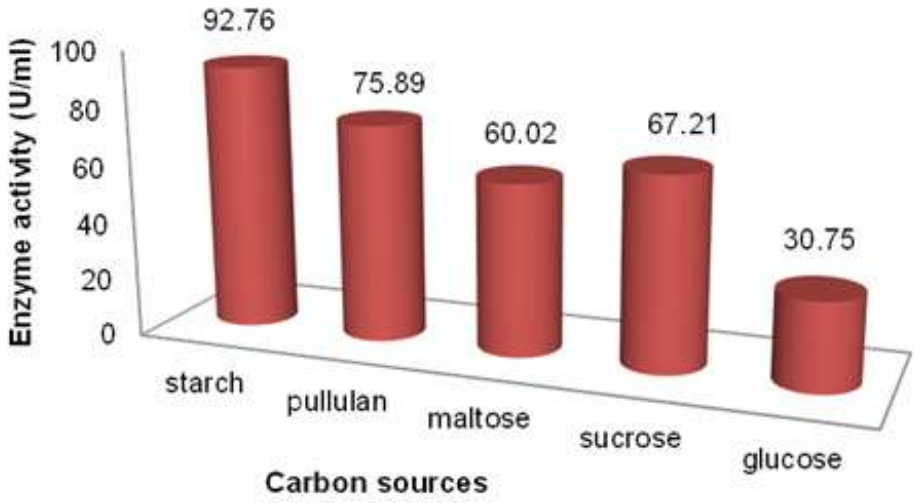

Figure 5. Effect of carbon sources on pullulanase production.

activity, while maltose, sucrose and glucose showed gradual decrease but significant in enzyme activity (Figure 5).

Effect of nitrogen sources

The effect of different nitrogen sources on the production of pullulanase was studied using the basal culture medium supplemented with 0.5% of nitrogen sources. Among the four organic nitrogen sources, peptone had more influence on pullulanase production (79.61 U/ml) than others (Figure 6). This may be due to the low molecular weight of peptone, which can be easily degraded and can be absorbed.

Different nitrogen sources were checked for enzyme activity among which peptone gave the maximum activity. The highest value of pullulanase activity was achieved in medium containing peptone which indicates that, peptone could be one of favorable organic nitrogen source that enhances the cell growth of the culture as well as the pullulanase production (Hii et al., 2009).

Determination of optimal conditions for pullulanase production in submerged fermentation (SmF)

The result depicted in Table 1 showed that, media containing starch as carbon source with peptone as nitrogen source, was inoculated with 1 ml of bacterial suspension when incubated at 37°C for 48 h at pH 7, which was the best media for pullulanase production.

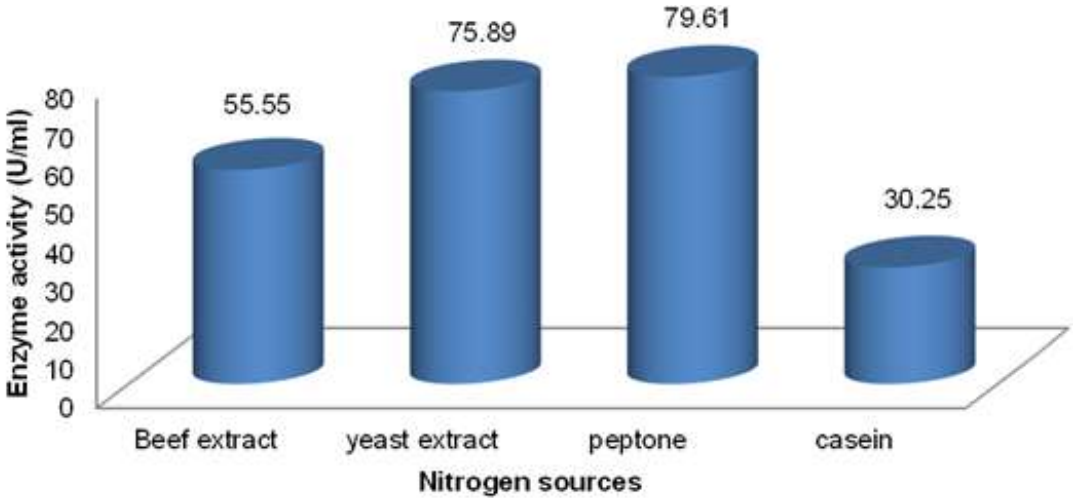

Figure 6. Effect of nitrogen sources on pullulanase production.

Table 1. Optimal conditions for pullulanase production by using submerged fermentation medium.

Parameters	Optimum for production	Enzyme activity (U/ml)
Carbon sources	Starch	92.76
Nitrogen sources	Peptone	79.61
Incubation period	48 h	78.62
Incubation temperature	37°C	90.53
pH	7.0	101.05

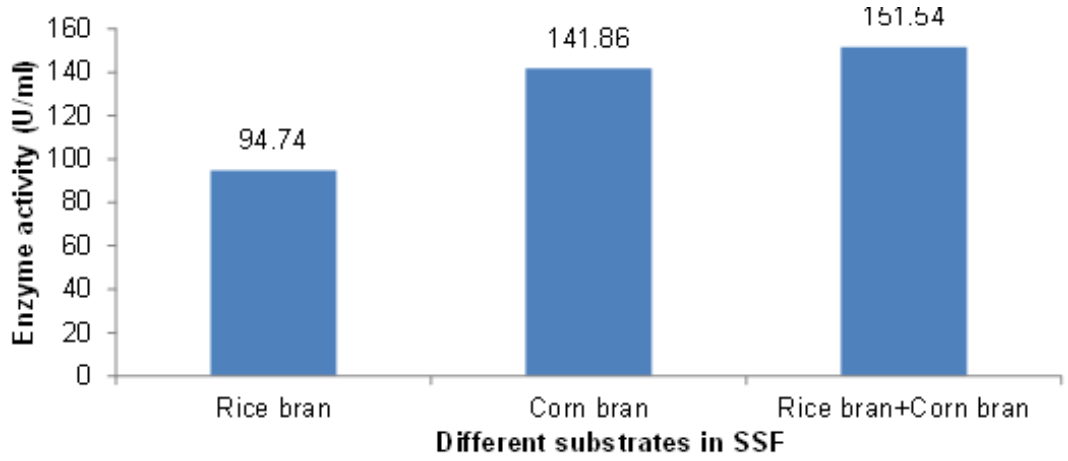

Figure 7. Enzyme activity of different sources in SSF.

Solid substrate fermentation (SSF)

Different solid substrates were used for pullulanase production from *K. aerogenes* NCIM 2239, as shown in Figure 7, which gave the highest enzyme activity in media containing mixture of rice bran and corn bran as substrate in SSF.

The enzyme activity reached 151.54 U/ml, while the media containing rice and corn bran gave the lowest enzyme activity. Similar results are also reported for

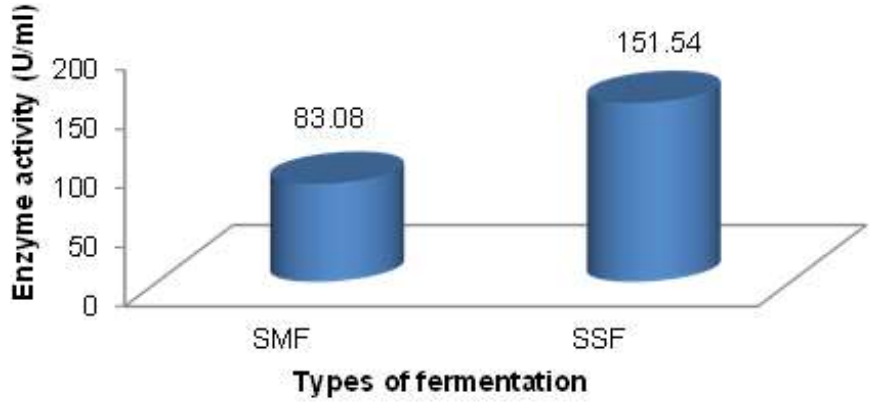

Figure 8. Comparisons between SSF and SmF.

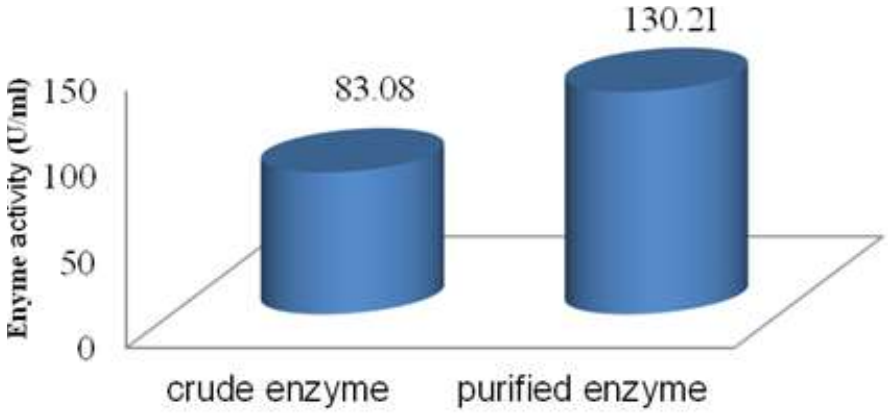

Figure 9. Enzyme activity between the crude and purified enzymes.

Bacillus lichenoformis (BS18) (Khalaf and Aldeen, 2013).

Comparison between SSF and SmF

SSF had given higher production of pullulanase than submerged fermentation (SmF). The results are shown in Figure 8.

SSF have been defined as the microbial transformation of biological materials in their natural state, in contrast with liquid or submerged fermentation that is carried out in dilute solution or slurries (Kotwal et al., 1998). The solid substrate in SSF provides a rich and complex source of nutrient, which may be sufficient or sometimes inadequate and incompete, with respect to the overall nutritional requirement of that particular microorganism that is cultivated on that substrate (Kotwal et al., 1998). SSF is preferred to SmF because of the simple technique, low capital investment, lower levels of catabolic repression and end product inhibition, low waste output, better product recovery, and high quality production (Mahanta et al., 2008).

Purification of crude enzyme by ultrafiltration method

The enzyme was purified by ultrafiltration method of membrane separation and enzyme activity, which was measured by DNSA method. The nature of solid substrate is the most important factor in SSF, which not only supplies the nutrient to the culture but also serves as an anchorage for microbial cells.

Therefore, the particle size and the chemical compositions of substrate are of critical importance (Kim et al., 1985) (Figure 9)

Conclusion

Pullulanase was successfully produced and purified from *K. aerogenes* NCIM2239 strain. As it is very stable at higher range of temperature and pH, this can be used in many industrial processes. The media preparation, which is one of the most important objective of this study was achieved and the most appropriate combinations of media components where stabilised.

Table 2. Enzyme activity between the crude and purified enzymes.

Parameters	OD at 540 nm	Concentration (µg/ml)	Enzyme activity (U/ml)
Crude enzyme	0.32	498.51	83.08
Purified enzyme	0.51	781.25	130.21

In order to study enzyme activity, the enzyme substrate reaction was performed based on various parameters such as temperature (37°C), incubation time (48 h) and pH 7. Various carbon sources were checked as media component among which starch gave best enzyme activity, while maltose, sucrose and glucose showed significant enzyme activity.

Peptone gave the maximum activity among different nitrogen sources. The study of the SSF medium, SmF and comparison between them indicated that, the use of SSF gave higher production of enzyme than SmF. SSF technique is a suitable and economic method for pullulanase production. The pullulanase activity of the crude form was 83.08 U/ml. The crude enzyme under purification using ultrafiltration gave rise to 130.21 U/ml enzyme activity, and thus can be used in various industrial processes (Table 2).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Ben Messaoud E, Ben Ammar Y, Mellouli L, Bejar S (2002). Thermostable pullulanase type I from new isolated Bacillus thermoleovorans US105: cloning, sequencing and expression of the gene in E. coli. Enzy. Microb. Technol. 31(6):827-832.

Bender H, Wallenfels K (1961). Investigation on pullulan II Specific degradation by means of a bacterial enzyme. Biochem. Z. 334:79-95.

Bertoldo C, Antranikian G (2002). Starch-hydrolyzing enzymes from thermophilic archaea and bacteria. Curr. Opinion Chem. Biol. 6:151-160.

Brown SH, Costantino HR, Kelly RM (1990). Characterization of amylolytic enzyme activities associated with the hyperthermophilic archaebacterium Pyrococcus furiosus. Appl. Environ. Microbiol. 56(7):1985-1991.

Brown SH, Kelly RM (1993). Characterization of amylolytic enzymes, having both α-1,4 and α-1,6 hydrolytic activity, from the thermophilic archaea Pyrococcus furiosus and Thermococcus litoralis. Appl. Environ. Microbiol. 59(8):2614-2621.

Catley BJ (1971). Utilization of carbon sources by Pullularia pullulans for the elaboration of extracellular polysaccharides. Appl. Microbiol. 22: 641-654.

Drummond GS, Smith EE, Whelan WJ (1969). Mechanism of action of pullulanase" FEBS Lett. 5:85-88.

Duan X, Wu J (2015). Enhancing the secretion 1 efficiency and thermostability of *Bacillus deramificans* pullulanase mutant D437H/ D503Y by N-terminal domain truncation. Appl Environ Microbiol. 81(6):1926-31.

Hii SL, Tau Ling TC, Mohamad R, Ariff AB (2009). Enhancement of Extracellular Pullulanase Production by Raoultella planticola DSMZ 4617 Using Optimized Medium Based on Sago Starch. The Open Biotechnol. J. 3:1-8.

Jensen BD, Norman BE (1984). *Bacillus acidopullyticus* pullulanase: applications and regulatory aspects for use in food industry. Proc. Biochem. 1:397-400.

Khalaf AK, Aldeen SB (2013). Optimum Condition of Pullulanase Production by Liquid State and Solid State Fermentation (SSF) Method from *Bacillus Licheniforms* (BS18). Iraq. J. Sci. 54:35- 49.

Kim CH, Nashiru O, Ko JH (1996). Purification and biochemical characterization of pullulanase type I from *Thermus caldophilus* GK-24. FEMS Microbiol. Lett. 38(2-3):147-152.

Kim JH, Hosobuchi M, Kishimoto M, Seki T, Ryu DDY (1985). Cellulase production by a solid state culture system. Biotechnol. Bioeng. 27:1445-1450.

Klingeberg M, Hippe H, Antranikian G (1990). Production of the novel pullulanases at high concentrations by two newly isolated thermophilic clostridia. FEMS Microb. Lett. 69(1-2):145-152.

Koch R, Canganella F, Hippe H, Jahnke KD, Antranikian G (1997). Purification and properties of a thermostable pullulanase from a newly isolated thermophilic anaerobic bacterium, Fervidobacterium pennavorans Ven5. Appl. Environ. Microbiol. 63(3):1088-1094.

Kornacker MG, Pugsley AP (1990). Molecular characterization of pulA and its product, pullulanase, a secreted enzyme of Klebsiella pneumoniae UNF5023. Mol. Microbiol. 4(1):73-85.

Kotwal SM, Gote MM, Sainkar SR, Khan MI, Khire JM (1998). Production of galactosidase by thermophilic fungus Humicola sp. in solid state fermentation and its application in soyamilk hydrolysis. Process Biochem. 33:337-43.

Ling HS, Ling TC, Rosfarizan, Ariff AB (2009) Characterization of Pullulanase type-II from Bacillus cereus H1.5. Am. J. Biochem. Biotechnol. 5:170-179.

Lonsane BK, Ghildyal NP, Budiatman S, Ramakrishna SV (1985). Engineering aspects of solid- state fermentation. Enzy. Microbial Technol. 7:258-265.

Mahanta N, Gupta A, Khare SK (2008). Production of protease and lipase by solvent tolerant Pseudomonas aeruginosa PseA in olid-state fermentation using Jatropha curcas seed cake as substrate. Bioresour Technol 99:1729-1735.

Ohba R, Ueda S (1973). Purification, crystallization and some properties of intracellular pullulanase from Aerobacter aerogenes. Agricul. Biol. Chem. 37(12):2821-2826.

Prakash N, Gupta S, Ansari M, Khan ZA, Suneetha V (2012). Production of economically important products by the use of pullulanase enzyme. Inter. J. Sci. Innov. Discov. 2(2):266-273.

Malakar R, Yadav M, Malviya SN, Tiwari A (2012). Effect of Media Composition and Culture Condition on Pullulanase Enzyme From Extremophile Bacterial Species. Int. J. Biomed. Adv. Res. 3(9):692-699.

Rudiger A, Jorgensen PL, G. Antranikian (1995), Isolation and characterization of a heat-stable pullulanase from the hyperthermophilic archaeon Pyrococcus woesei after cloning and expression of its gene in Escherichia coli. Appl. Environ. Microbiol. 61(2):567-575.

Shaw JF, Sheu JR (1992). Production of high-maltose syrup and high-protein flour from rice by an enzymatic method. Biosci. Biotechnol. Biochem. 56(7):1071-1073.

Shehata AN, Darwish DA, Masoud HMM (2016). Extraction, Purification and Characterization of Endo-Acting Pullulanase Type I from White Edible Mushrooms. J. Appl. Pharm. Sci. 6(01):147-152.

Hii SL, Tan JS, Ling TC, Ariff AB (2012). Pullulanase: Role in Starch Hydrolysis and Potential Industrial Applications. Enzy. Res. 2012(921362):1-14.

Sunna A, Moracci M, Rossi M, Antranikian G (1997). Glycosyl hydrolases from hyperthermophiles. Extremophiles 1(1):2-13.

Suzuki Y, Hatagaki K, Oda H (1991). A hyperthermostable pullulanase produced by an extreme thermophile, *Bacillus flavocaldarius* KP 1228, and evidence for the proline theory of increasing protein thermostability. Appl. Microbiol. Biotechnol. 34(6):707-714.

Swamy MV, Seenayya G (1996). Thermostable pullulanase and a-amylase from Clostridium thermosulfurogenes SV9: Optimization of culture conditions for enzymes production. Process Biochem. 31:157-162.

Takasaki Y, Hayashida A, Ino Y, Ogawa T, Hayashi S, Imada K(1993). Cell-bound pullulanase from Streptomyces sp. No. 27. Biosci. Biotechnol. Biochem. 57(3):477-478.

Waleed M, Faiza AA, Nizar I (2015). Production optimization of pullulanase enzyme produced by *Bacillus cereus* isolated from Syrian sources. Inter. Food Res. J. 22(5):1824-1830.

Murooka Y, Ikeda R (1989). Biosynthesis and secretion of pullulanase, a lipoprotein from Klebsiella aerogenes. J. Biol. Chem. 264(29):17524-17531.

Zhu Z-P, Hylton CH, Rossner U, Smith AM (1998) Characterization of Starch-Debranching Enzymes in Pea Embryos. Plant Physiol. 118:581-590.

Liu Z, Deng X, Wang M, Chen J, Zhang A, Gu Z, Zhao C (2009). BSA-Modified Polyethersulfone Membrane: Preparation, Characterization and Biocompatibility. J. Biomat. Sci. 20:377-397.