An Elsevier Indexed Journal ISSN-2230-7346

Journal of Global Trends in Pharmaceutical Sciences

ENRICHMENT OF EXTRACELLULAR PULLULANASE PRODUCTION BY ISOLATED *BACILLUS CEREUS* KKSJ 1981 THROUGH OPTIMIZATION OF FERMENTATION CONDITIONS

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ARTICLE INFO

Key Words
Glycoside hydrolases, starch degradation, Optimization, Pullulanase, one factor-at-a-time method and biofuels.

ABSTRACT

Pullulanases are the enzymes which convert the polysaccharides to oligosaccharides. These enzymes have industrial importance specifically in starch based companies. Production of pullulanase at cost-competitive methods are inspiring to exploration of new microbial sources and optimization of various fermentation process parameters for enhancement of enzyme yields. In the present study pullulanase was produced by isolated *Bacillus cereus* KKSJ 1981 and various process parameters such as incubation time (hrs), initial pH, incubation temperature (°C), agitation rate (rpm), amount of inoculum (%) and nutritional sources such as carbon, nitrogen and minerals were optimized by one factor at a time method. By optimization pullulanase production was enhanced from 3.7 U/ml to 10.31 U/ml. An overall ~1.8 fold of pullulanase production by *Bacillus cereus* KKSJ 1981was enhanced by optimization of various fermentation parameters.

INTRODUCTION

Pullulanase (pullulan 6-glucanohydrolase; EC 3.2.1.41) which is an important enzyme in the Glycoside hydrolases (GHs). GHs have played a vital role in various biological processes such as cell wall metabolism, degradations and biosynthesis of glycan. Among various GHs amylases and pullulanases have specific industrial importance (Guan *et al.* 2013). Pullulanase hydrolyzes pullulan as maltotriose units by attacking the α-1,6-glycosidic linkages in the polysaccharide. Based on their debranching of oligosaccharides these enzymes are classified as type I & type II pullulanases. Type I pullulanases Specifically attacks on α-1,6-glycosidic linkages in carbohydrates whereas type II pullulanases cleaves both α-1,6 and α-1,4-glycosidic linkages in pullulan, amylopectin, starch, and other related polysaccharides (Guan *et al.* 2013; Nielsen, 2000; Doman-Pytka and Bardowski 2004; Bertoldo and Antranikian 2002). According to Ren *et al.* (2018) increasing the global population fetches the depletion and shortfall of resources like fuels and increases the environmental pollution (Wang *et al.* 2019). Hence technologies are needed which brings sustainable development and environmental protection. Keeping this in view many governments are recommending the plant biomass based technologies. Plant biomass such as agricultural or non-agricultural

biomass could be converted into biofuels, fine chemicals and other useful products (Prakasham *et al.* 2010). However chemical and physical processes of conversion of biomass are high energy intensive methods as well as release toxic byproducts. Conversion of biomass by using biocatalysts has more advantages such as low energy consumption, economically viable and minimal pollution (Laxmi *et al.* 2008). Starch and cellulose are the major compounds present in the plant biomass. For centuries starch has been using as raw material for food, textile, distillery, pharma and detergent industries. Based on the origin of starch they have various amylose to amylopectin ratios (Laxmi *et al.* 2008). Effective conversion of starch into sugars and oligosaccharides are the crucial step in the starch based industries. Primarily amylases are considered as the potent enzymes which convert the starch into dextrans, however most of the amylases can cleave α-1,4-glycosidic linkages in the starch. The enzyme like pullulanase which cleaves the α-1,4 and α-1,6-glycosidic linkage in the starch and totally converts it into simple sugars (Wang *et al.* 2019). Among various sources of pullulanases, bacterial sources have gained importance because of their easy cultivation and recovery of enzyme (Nair *et al.* 2006). Former reports denotes that *Bacillus* sp. (Teague *et al.* 1992; Uhlig and Linsmaier-Bednar, 1998; Nair *et al.* 2007) mainly *Bacillus cereus* (Bakshi*et al.* 1992; Nair *et al.* 2007; Waleed *et al* 2015; Sathish and Prakasham 2012) are the predominantly extracellular pullulanase producing bacteria. In the present study pullulanase production studies were carried out by using isolated *Bacillus cereus* KKSJ 1981. To enhance the production of pullulanase various environmental and nutritional parameters were optimized.

MATERIALS AND METHODS:

Microorganism and inoculum preparation: The isolated *Bacillus cereus* KKSJ 1981 (MN592984) was used in this study. The culture was stored on nutrient agar slants. A routine subculture was carried out. For inoculum preparation, a loop full of microorganism transferred into 50 ml of nutrient broth and incubated at 37°C on a shaker incubator. Once the culture achieves 0.8 OD at 600 nm (~10^8 cells/ml) it is used to prepare the inoculum. For optimization studies 18 to 24 hrs actively growing culture (~10^8 cells/ml) was used.

Production of pullulanase: The extracellular pullulanase was produced from *B. cereus* KKSJ 1981 in pullulan media (Waleed *et al.* 2015). The media consist of pullulan 10 g/L, Nacl 2 g/L, $MgSO_4$ 0.1 g/L, K_2HPO_4 0.17 g/L, KH_2PO_4 0.12 g/L and pH 7.5. The media was sterilized at 121°C for 20 min and inoculated with 1% inoculum, incubated at 37°C for 72 hrs. After incubation, the broth was centrifuged at 10,000 rpm for 10 min at 4°C temperature. The cell's free broth was considered as a crude enzyme and assayed for pullulanase activity.

Assay of Pullulanase enzyme activity: The pullulanase enzyme activity was estimated according to Ara *et al.* (1995). The enzyme activity was measured by using pullulan as a substrate. The reaction mixture consists of 1 % pullulan in 460 µL of 0.05M phosphate buffer, pH 7. To this add 40 µL of crude extract, incubated at 40°C for 10 min. The reducing sugar released was measured using DNS (Dinitrosalicylic acid) method (Miller, 1959). Pullulanase Activity was defined as the amount of µmols of D-glucose released per minute under assay conditions and it was expressed in U/ml.

Optimization of pullulanase production: All parameters were optimized by one-at-a-time method. In this, only one parameter was optimized at a time and the optimum conditions were used in the subsequent experiments. Experiments were conducted in a 250 ml flask with 100 ml sterile media. The data presented is the mean of triplicates. The following parameters were optimized.

Effect of incubation Period on pullulanase production: In order to study the effect of the incubation period the flasks were incubated up to 72 h. Every 12 h samples were drawn and analyzed for enzyme activity.

Effect of pH and Temperature on pullulanase production: To study the initial media pH on pullulanase production by *B. cereus* KKSJ 1981 was analyzed by adjusting

the media pH from 3.5 to 9. The incubation temperature effect on pullulanase production by *B. cereus* KKSJ 1981 was studied from 20-40°C by adjusting the shaker incubator temperature. The flasks were harvested at a particular temperature and enzyme titers were measured.

Effect of agitation speed and Inoculum level on Pullulanase production: In the direction of optimization of agitation speed on pullulanase production by *B. cereus* KKSJ 1981 the media with culture flasks was incubated at different rpm levels from 50 to 250 with increment of 25.To study the effect of inoculum levels on pullulanase production by *B. cereus* KKSJ 1981, different amounts of inoculum (10^6 cfu/ml) from 0.5 to 3.5 % was added to various flasks. The flasks were harvested at optimum temperature and the pullulanase activity was measured. The optimum rpm & inoculum level achieved in these experiments was used in subsequent experiments.

Effect of Carbon & Nitrogen source on Pullulanase production: The carbon source plays a vital role in the rate of pullulanase production. To study the effect of various carbon sources such as Pullulan, Soluble starch, Potato starch, Tapioca Starch, Amylopectin, Glycogen, Maltose, Amylose, Xylose, Lactose, Sucrose, Glucose, Mannitol, Fructose and Galactose on secretion of pullulanase by *B. cereus* KKSJ 1981 all compounds were tested at 1 % in the media. Only one carbon source added to the media. The nitrogen sources such as Tryptone, Peptone, Casein, Yeast extract, Beef Extract, Malt Extract, Corn steep Liquor, Soya flour, NH_4Cl, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, $NaNO_3$, and combination of yeast extract and tryptone were also studied. Unlike carbon sources, the studied nitrogen sources were taken at 0.5% concentration.

Effect of Minerals on Pullulanase production: To study the minerals effect on pullulanase secretion by *B. cereus* KKSJ 1981, different minerals were added to the media at 0.5mM concentration. The minerals tested in this study were MnSO4, $MgCl_2$, NaCl, Na_2HPO_4, KH_2PO_4, K_2HPO_4, $ZnSO_4$, $FeSO_4$, $CaCl_2$, Na_2CO_3 and $BaCl_2$.

RESULTS AND DISCUSSION:

Pullulanases play a vital role in starch based industries. However biofuels and other products from the starch industry have low market value. Sustainable growth of these industries depends on the cost-competitive methods of starch saccharification. Production of the enzyme by low cost procedures are highly recommended. Fermentation plays a vital role in the quality and final yield of the enzymes (Sathish and Prakasham 2011). Further many researchers have achieved an increased Pullulanases production by altering the fermentation process and environmental parameters (Nair *et al.* 2006; Waleed *et al.* 2015).Keeping this in view various fermentation parameters were optimized for enhancement of pullulanase production by *B. cereus* KKSJ 1981.

Effect of incubation time on pullulanase production: The effect of incubation time on pullulanase production by *B. cereus* KKSJ 1981 was observed at 12-72 hrs with 12hr interval. It was observed that the production of enzyme was increased from 12 hrs to 48 hrs (Fig 1). The maximum enzyme production was observed at 48hrs (3.7 U/ml). At 60 hrs the production was decreased to 1.32 U/ml and at 72hrs no enzyme production was observed. The current results indicates that the extracellular enzyme production was increased until the microorganism reached to the stationary phase. After that, the production was decreased. The reduced enzyme production was attributed by depletion of nutrients in the media that initiates the secretion of proteases in the media. Nair *et al*. (2006) and Waleed *et al*. (2015) reported the similar results from *Bacillus cereus*. Khalaf and Aldeen (2013) also observed highest pullulanase at 48 hrs by *Bacillus* sp. however Asha *et al*. 2013 reported that high level of pullulanase production by *Bacillus halodurans* was achieved after 144 hrs incubation period. Prabhu *et al*. (2018) reported that they obtained the maximum pullulanase production at 48 hrs from *Klebsilla aerogenes* NCIM 2239. Based on literature reports and current experimental results it was observed that the pullulanase secretion depends on medium composition, culture conditions and microbial strain.

Effect of pH and Temperature on pullulanase production: Fig 2 depicts the role of the initial media pH on pullulanase secretion by *B. cereus* KKSJ 1981. From this figure it was noticed that the maximum enzyme production was obtained at pH 7, below and above this pH decreased production was

observed. The pullulanase production was studied at the pH range 3.5 to 9 shows an inverted parabola pattern, from pH 4.5 enzyme secretion was increased and it was reached to maximum at pH 7 and from that it was decreased, after pH 8 severe fall of production was noticed. The obtained results are in agreement with the literature reports on pullulanase enzyme production from *B. cereus*. Nair *et al*. (2006) and Waleed *et al*. (2015) reported that they observed maximum pullulanase enzyme production at pH 6.5 and 7.5. Mrudula (2011) reported that pullulanase production was maximum at pH 7 for *Clostridium thermosulfurogenes* SVM17. Prabhu *et al*. (2018) reported that maximum pullulanase production was noticed at pH 7 from *Klebsilla aerogenes* NCIM 2239. Incubation temperature plays a vital role in the production of extra cellular enzymes from *Bacillus* sp. (Sathish *et al*. 2018). In the present study to know the optimum incubation temperature for the maximum yield of pullulanase from *B. cereus* KKSJ 1981, the media with culture was incubated at 20 to 40 °C. From 28°C a noticeable amount of pullulanase production was observed and it reached to the maximum at 32°C (Fig 3). Above this temperature, the enzyme production was decreased. After 36°C incubation temperature sharp fall of enzyme production was noticed. The obtained results are in accordance with Nair *et al*. (2006), they kept incubation temperature as 32±2°C for pullulanase production from *B. cereus*. However Waleed *et al*. (2015) reported that they observed higher amounts of enzyme production at 37°C from *B. cereus*. Similarly Asha (2013) reported that *B. Halodurans* was capable of producing pullulanase in the range of 28–65°C with maximum production at 37°C (Waleed et al 2015). Prabhu *et al*. (2018) also reported that *Klebsiella aerogenes* NCIM 2239 produces higher amounts of pullulanase at 37°C.

Effect of Agitation speed and Inoculum level on Pullulanase production: Mixing plays a vital role in the release of extracellular enzymes production in the submerged fermentation (Sathish *et al*. 2018). Fig 4 depicts the role of agitation on pullulanase production by *B. cereus* KKSJ 1981. From this figure it was observed that at 200 rpm the highest enzyme production (5.25 U/ml) was noticed. Above or below this rpm decreased production was observed. The obtained results were in accordance with Nair *et al*. (2006) produced pullulanase by *B. cereus*. The amount of inoculum plays an important role in the production of pullulanase (Waleed *et al*. 2015). Less enzyme production was observed at lower inoculum level might be because of prolonged lag phase. Similarly higher amount of inoculum also shows decreased enzyme production due to decline in log phase, attains the stationary phase, decreases the nutrients in the medium leads to accumulation of large quantities of toxins into the media. To optimize initial microbial load on pullulanase production by *B. cereus* KKSJ 1981, 0.5 to 3.5 % of inoculum was added to the sterile media. It was observed that 2 % of inoculum yielded the maximum pullulanase (6.98 U/ml) (Fig 5). Deviance from this inoculum concentration decreased enzyme production was noticed. The observed inoculum level is lesser than the Nair *et al*. (2006) and closer to the Waleed *et al*. (2015) studies on pullulanase production from *B. cereus*.

Effect of Carbon & Nitrogen source on Pullulanase production: The carbon source in the media plays a vital role in pullulanase production, since it acts as a nutrient and inducing agent for enzyme secretion from microorganisms. Some of the carbon sources and their concentrations also act as catabolic repressors (Nair *et al*. 2006). In the present study pullulan, added media was considered as a control. Soluble starch, potato starch, tapioca starch and amylopectin enhanced the pullulanase production by *B. cereus* KKSJ 1981. Among all studied carbon sources soluble starch yielded the maximum production of pullulanase (Fig 6). Maximum production of pullulanase was noticed with soluble starch, which increased the yield 26 % as compared to control. The monosaccharides and disaccharides repressed the enzyme production. Glucose suppressed the 86% of pullulanase production compared with control. Enhanced pullulanase production with soluble starch could be attributed to the presence of α-(1,6)-linkages, present in complex polysaccharides that can induce the production of pullulanase (Suzuki & Chishiro 1983; Antranikian 1990; Nair *et al*. 2006).Similar outcomes were

noticed with Nair et al(2006) an increased pullulanase synthesis by soluble starch in *B. cereus.*

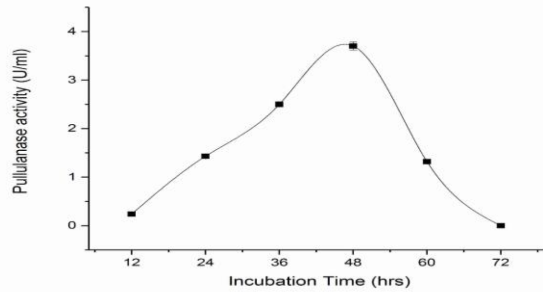

Fig 1: Effect of Incubation period on Pullulanase production by *B. cereus* KKSJ 1981.

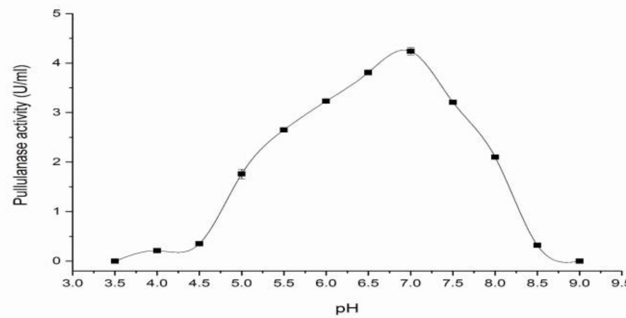

Fig 2: Effect of media pH on Pullulanase production by *B. cereus* KKSJ 1981.

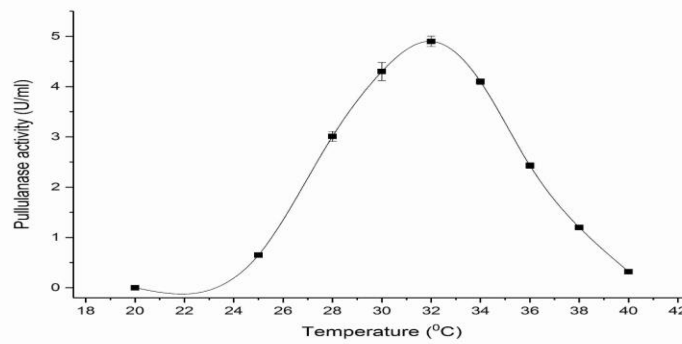

Fig 3: Effect of incubation temperature on Pullulanase production by *B. cereus* KKSJ 1981.

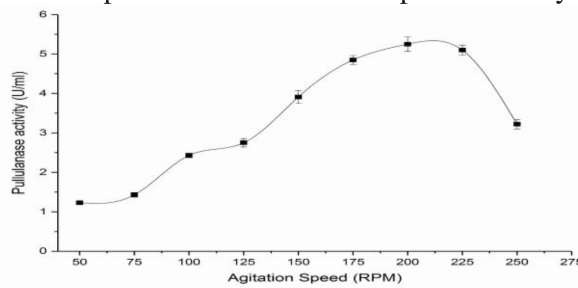

Fig 4: Effect of Agitation on Pullulanase production by *B. cereus* KKSJ 1981.

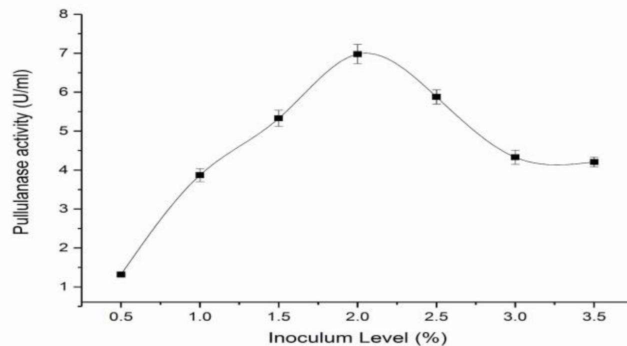

Fig 5: Effect of Inoculum concentration on Pullulanase production by *B. cereus* KKSJ 1981.

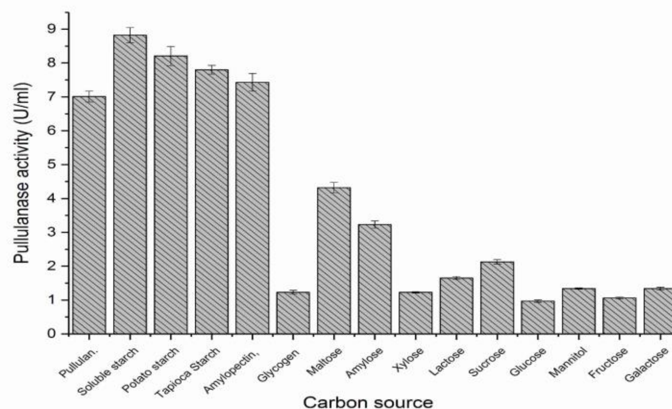

Fig 6: Effect of Carbon source on Pullulanase production by *B. cereus* KKSJ 1981.

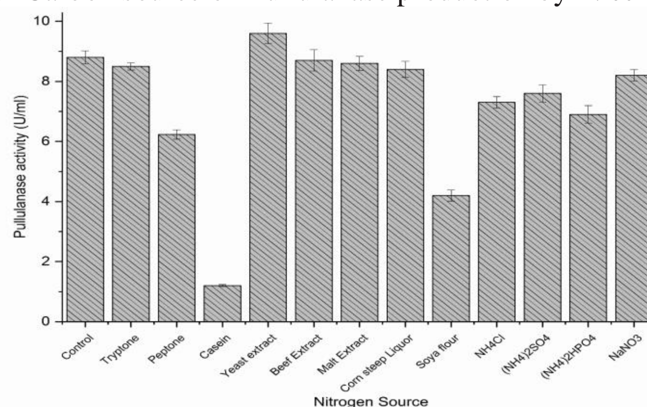

Fig 7: Effect of Nitrogen source on Pullulanase production by *B. cereus* KKSJ 1981.

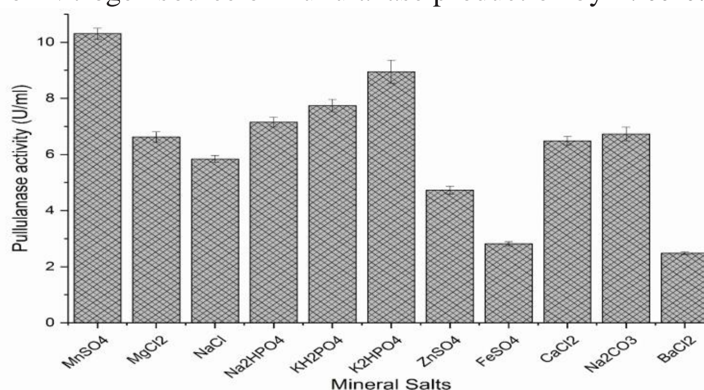

Fig 8: Effect of Minerals supplement on Pullulanase production by *B. cereus* KKSJ 1981.

However, the authors are contradicting in enhancement of pullulanase production with maltose supplement. Maltose suppressed pullulanase production was noticed in *Clostridium thermohydrosulfuricum* (Melasniemi 1987). Asha *et al*. (2013) and Prabhu *et al*. (2018) also reported that soluble starch enhanced pullulanase production by *B. halodurans* and *K. aerogenes* NCIM 2239 respectively. To study the effect of nitrogen source on the pullulanase production by *B. cereus* KKSJ 1981, a total 12 organic, inorganic nitrogen compounds and combination of yeast extract and tryptone was studied (Fig 7). Among all tested compounds yeast extract supports higher amounts (9.6 U/ml) of pullulanase production by *B. cereus* KKSJ 1981. Tryptone, peptone and other studied nitrogen sources not enhanced enzyme activity. Lowest enzyme activity was noticed with casein supplementation media. It was observed that casein triggered the protease production as an alternative to pullulanase by *B. cereus* KKSJ 1981 (data not shown). The inorganic nitrogen sources decreased the enzyme yield this could be attributed due to release of ions in the

media. Among studied inorganic nitrogen sources $NaNO_3$ yielded a higher amount of pullulanase than ammonium salts. The combination of yeast extract and tryptone has not increased the enzyme yield as yeast extract is a solo supplement. The obtained results are in accordance with Nair *et al.* (2006) produced pullulanase from *B. cereus*. Waleed *et al.* (2015) noticed that supplementation of tryptone and peptone produced maximum pullulanase from *B. cereus*. It also reported that *Klebsiella aerogenes* NCIM 2239 (Prabhu *et al.* 2018), *B. halodurans* (Asha *et al.* 2013) and *Clostridium thermosulfurogenes* SV9 (Swamy and Seenayya, 1996) yields more amount of pullulanase with tryptone and peptone supplementation.

Effect of Minerals on Pullulanase production: Fig 8 shows the effect of minerals supplementation on pullulanase production by *B. cereus* KKSJ 1981. From this figure it was observed that among all studied minerals $MnSO_4$ enhanced enzyme activity over the control other minerals suppressed the enzyme production. $FeSO_4$ and $BaCl_2$ has the highest inhibition effect on pullulanase production by *B. cereus* KKSJ 1981. Takasaki *et al.* (1976) and Nair *et al.* (2006) observed similar results produced pullulanase by *Bacillus* sp and *B. cereus* respectively.

CONCLUSION:

The results of the present study describe the effect of various fermentation parameters on *B. cereus* KKSJ 1981 for the enhancement of enzyme yields. Production of pullulanase by *B. cereus* KKSJ 1981 was enhanced from 3.7 U/ml to 10.31 U/ml by optimization of various fermentation parameters such as incubation time (hrs), initial pH, incubation temperature (°C), agitation rate (rpm), amount of inoculum (%) and nutritional sources such as carbon, nitrogen and minerals. *B. cereus* KKSJ 1981can be a potential producer for extracellular pullulanase enzyme.

REFERENCES:

1. Antranikian G. Physiology and enzymology of thermophilic anaerobic bacteria degrading starch. FEMS Microbiology Reviews. 1990; 75: 201–218.
2. Ara K, Saeki K, Igarashi K, Takaiwa M, Uemura T, Hagihara H, Kawai S, Ito S. Purification and characterization of an alkaline amylopullulanase with both alpha-1,4 and alpha-1,6 hydrolytic activity from alkalophilic *Bacillus* sp. KSM-1378. Biochimica et Biophysica Acta 1995; 1243: 315–324.
3. Asha R, Niyonzima FN, Sunil SM. Purification and Properties of Pullulanase from *Bacillus halodurans*. International Research Journal of Biological Sciences. 2013; 2(3): 35–43.
4. Bakshi A, Gupta JK, Patnaik PR. Pullulanase and α-amylase production by a *Bacillus cereu*s isolate. Letters in Applied Microbiology 1992; 14: 210–213.
5. Bertoldo C, Antranikian G. Starch-hydrolyzing enzymes from thermophilic archaea and bacteria. Current Opinion in Chemcal Biology 2002; 6: 151–60.
6. Doman-Pytka M, Bardowski J. Pullulan degrading enzymes of bacterial origin. Critical Reviews in Microbiology. 2004; 30: 107–121.
7. Guan Q, Guo X, Han T, Wei M, Jin M, Zeng F, Liu L, Li Z, Wang Y, Cheong GW, Zhang S, Jia B. Cloning, purification and biochemical characterisation of an organic solvent, detergent, and thermo-stable amylopullulanase from *Thermococcus kodakarensis* KOD1. Process Biochemistry. 2013; 48: 878–884.
8. Khalaf AK, Aldeen SB. Optimum Condition of Pullulanase Production by Liquid State and Solid State Fermentation (SSF) Method from Bacillus Licheniforms (BS18). Iraq Journal of Science. 2013; 54: 35–49.
9. Laxmi GS, Sathish T, Subba Rao Ch, Brahmaiah P, Hymavathi M, Prakasham RS. Palm fiber as novel substrate for enhanced xylanase production by isolated *Aspergillus* sp. RSP-6. Current Trends of Biotechnology and Pharmacy. 2008; 2: 447–455.
10. Melasniemi H. Characterization of a-amylase and pullulanase activities of *Clostridium thermohydrosulfuricum*–Evidence for a novel thermostable

amylase. Biochemical Journal. 1987; 246: 193–197.

11. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry. 1959; 31: 426-428.
12. Mrudula S, Reddy G, Seenayya G. Screening of Various Raw Starches on Production of Thermostable Amylopullulanase by *Clostridium thermosulfurogenes* SVM17. World Applied Sciences Journal 2011; 15: 996–1001.
13. Nair SU, Singhal RS, Kamat MY. Enhanced Production of Thermostable Pullulanase Type 1 Using *Bacillus cereus* FDTA 13 and Its Mutant. Food Technology and Biotechnology. 2006; 44(2): 275–282.
14. Nair SU, Singhal RS, Kamat MY. Induction of pullulanase production in *Bacillus cereus* FDTA- 13. Bioresource Technology. 2007; 98: 856–859.
15. Nielsen JE, Borchert TV. Protein engineering of bacterial α-amylases. Biochimcal Biophyssical Acta 2000;1543:253–274.
16. Prabhu N, Maheswari RU, Singh MVP, Karunakaran S, Kaliappan C, Gajendran T. Production and purification of extracellular pullulanase by *Klebsilla aerogenes* NCIM 2239 African Journal of Biotechnology. 2018; 17(14): 486-494.
17. Prakasham RS, Sathish T, Brahmaiah P. Biohydrogen production process optimization using anaerobic mixed consortia: A prelude study for use of agro-industrial material hydrolysate as substrate. Bioresource Technology. 2010; 101: 5708–5711.
18. Ren D, Yang Y, Yang Y, Richards K, Zhou X. Land-Water-Food Nexus and indications of crop adjustment for water shortage solution. Science to the Total Environment. 2018; 626: 11–21.
19. Sathish T, Prakasham RS. Effect of various environmental parameters on secretion of L-Glutaminase from *Bacillus subtilis* RSP-GLU. International Journal of Pharmceutical Chemical Sciences. 2012; 1: 625-631.
20. Sathish T, Kezia D, Bramhachari PV, Prakasham RS. Multi-objective based superimposed optimization method for enhancement of L-glutaminase Production by *Bacillus subtilis* RSP-GLU. Karbala International Journal of Modern Science. 2018; 4(1), 50–60.
21. Sathish T, Prakasham RS. Modeling the Effect of L-Glutamine, Aeration and Agitation Regimes on Production of L-Glutaminase in Stirred Tank Reactor using *Bacillus subtilis* RSP-GLU. International Journal of Industrial Biotechnology. 2011; 1: 27–33.
22. Suzuki Y, Chishiro M. Production of extracellular thermostable pullulanase by an amylolytic obligately thermophilic soil bacterium, *Bacillus stearothermophilus* KP-1064. European Journal of Applied Microbiology and Biotechnology. 1983; 17: 24–29.
23. Swamy MV, Seenayya G. Thermostable pullulanase and α-amylase activity from *Clostridium thermosulfurogenes* SV9—Optimization of culture conditions for enzyme production. Process Biochemistry. 1996; 31: 157-162.
24. Takasaki Y. Production and utilizations of b-amylase and pullulanase from *Bacillus cereus* var. *mycoide,* Agricultural Biological Chemistry.1976; 40: 1515–1522.
25. Teague W, Brumm P, Schenk F, Hebeda R. Commercial enzymes for starch hydrolysis products. VCH Publishers. pp: 45-78, 1992.
26. Uhlig H, Linsmaier-Bednar EM. Industrial enzymes and their applications. Wiley, pp: 89–93, 96–99, 226–227, 1998.
27. Waleed M, Faiza AA, Nizar I. Production optimization of pullulanase enzyme produced by Bacillus cereus isolated from Syrian sources. International Food Research Journal. 2015; 22(5): 1824-1830.
28. Wang X, Nie Y, Xu Y. Industrially produced pullulanases with thermostability: Discovery, engineering, and heterologous expression. Bioresource Technology. 2019; 278: 360-371.