Production of extracellular Pullulanase by NP 7 bacteria using OFAT methodology

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Abstract

Pullulanase is a debranching enzyme that spilts explicitly α-1,6 glycosidic bonds. It is widely used in starch saccharification, in production of glucose, fructose as well as maltotriose syrup and bioethanol production. Pullulanase is produced by submerged fermentation process that requires optimization of physical and chemical parameters to achieve higher activity and make this process cost effective. The present study aimed the optimization of physical as well as chemical parameters of production medium. The optimization includes physical parameters such as inoculum size, incubation time, temperature, pH and agitation rate while chemical parameters include carbon source, nitrogen source and different metal ions.

From the optimization study, it was found that highest pullulanase production was achieved after 48 hours of incubation at 45ºC temperature, pH 7 and 120 rpm. Optimization of chemical parameters reveals that incorporation of starch (1%) and 0.2mMole/L of calcium chloride in the media gave significant increase in enzyme activity. The maximum productivity achieved after optimization was 139.33 U/ml using OFAT method which was four times higher.

Keywords: Pullulanase, saccharification, calcium chloride, starch, pH.

Introduction

Pullulanase (pullulan-6-glucanohydrolase, EC:3.2.1.41) is a debranching enzyme that specifically hydrolyses the α-1,6-glycosidic linkages in pullulan and other branched polysaccharides such as starch[8] which can lower the dosage of glucoamylase, reduces the reaction time and efficiently improves the conversion rate[7,23]. Pullulanase hydrolyses pullulan, an α-glucan elaborated by *Pullularia pullulans* into maltotriose units, although traces of higher polysaccharides have also been detected[8]. Bacterial Pullulanase was isolated from *Thermotoga neapolitana, Geobacillus thermocatenulatus, Bacillus acidopullulyticus, Thermococcus marinus* and *Klebsiella variicola*[5] in recent years. To increase the efficiency and yield of enzymatic reactions with amylolytic enzymes like α-amylase, β-amylase or glucoamylase, addition of pullulanase is the most important in starch processing. Pullulanase enhanced the quality of sugar syrup in the starch saccharification process[9]. In food and pharmaceutical industries, pullulanase hydrolysed the products of pullulan which are frequently used[23]. Pullulanase also improves the production of cyclodextrins by pre-treating the starch prior to the cyclodextrin glucanotransferase (CGTase) reaction[12]. Pullulanase also has applications in the textile, baking and detergent industries[10].

Mesophilic, thermophilic and hyperthermophilic bacteria have ability to produce several pullulanase in high amounts[9]. Due to wide application of pullulanase in industry, several studies have been done to enhance enzyme production in bacterial cells by optimizing medium composition as well as physical parameters. The most common parameters are the types of carbon and nitrogen sources used in the fermentation medium. Pullulan, sucrose, maltose, glucose and starch are types of carbon sources previously used to improve pullulanase production[13,14].

Additionally, nitrogen sources, including yeast extract, peptone, tryptone, casein, ammonium acetate, ammonium sulphate and sodium nitrate, were found to improve the pullulanase production[24].

- Microorganisms require optimum production condition and nutrient to achieve maximum productivity of enzyme. Moreover, media optimization should be carried out to get specific media and condition which is cost effective as submerged fermentation is expensive.
- In the current study, media optimization was carried out using OFAT to achieve higher productivity of enzyme by pullulanase producing bacterial strain NP 7.

Material and Methods

Bacterial culture: For isolation of NP 7 bacterial strain, Soil sample (5 g) was added to 50 ml of medium containing soluble starch 10g/L, yeast extract 0.6g/L, K_2HPo_4 5 g/L, NaCl 1 g/L, $MgSO_4$ 0.2 g/L, $(NH_4)_2SO_4$ 0.6 g/L (pH 7.0) and incubated at 37ºC for 5 days. 0.2 ml of sample was spread on nutrient agar plate and incubated at 37ºC for 24 to 48 hours. For screening of pullulanase producing microorganisms, a medium (pH 7.0) was required containing pullulan-5, peptone-8, NaCl-2, $MgSO_4$-0.1, K_2HPO_4-0.17, KH_2PO_4-0.12 (g/L). Pullulanase producers were further screened on different concentrations of pullulan using plate method[7]. The pullulanase producers were isolated at 37º C using pH 7.0. Pullulanase producing miroorganisms were preserved on 0.5% pullulan containing basal medium (Pullulan-5, Peptone-8, NaCl-2, $MgSO_4$-0.1, K_2HPO_4-0.17, KH_2PO_4-0.12 (g/L) pH-7.0) at 4ºC.

Pullulanase production in submerged fermentation
Seed culture: 24-hour-old actively growing bacterial culture was inoculated in 250ml conical flask containing 50ml of 0.5% pullulan containing basal broth. The inoculum medium was incubated at 37 °C in rotary shaking at 120 rpm for 24h.

Pullulanase Production: The turbidity in terms of absorbance was adjusted to ~2.0 at 660 nm and production flasks were inoculated with 1% bacterial culture. Fermentation was carried out in 250-mL flasks containing 100 mL of pullulan medium at 37°C upto 48 hrs at 120 rpm. After interval of 24 hours, samples were harvested and centrifuged at 7500 rpm for 10 min at 4°C. The supernatant was used for determination of pullulanase enzyme activity[23].

Pullulanase enzyme assay: Pullulanase activity was assayed by measuring the reducing sugar (glucose) released from pullulan. The reaction mixture (1ml) containing pullulan (0.5% w/v) in sodium phosphate buffer (0.02 M, pH 7.5) and enzyme was incubated at 40°C for 30 min. The reducing sugar was measured by the dinitrosalicylic acid method according to Miller[17].

The blank contained all the assay constituents except the active enzyme which was replaced by buffer. One unit of pullulanase activity is defined as the amount of enzyme which produces 1µmol of reducing sugar with glucose as standard per min under the optimum conditions [2].

Characterization of crude pullulanase production: The cell free extract of the bacteria was taken as the crude pullulanase preparation. While studying each parameter, the other reaction conditions were kept constant and after every 24 hours, pullulanase activity was measured up to 72 hours.

Optimization of physical parameters
Effect of inoculum size on pullulanase production: Different concentrations of seed inoculum were inoculated in production medium to check the optimum concentration of culture to achieve higher production.

Different inoculation sizes ranging from 1%, 2 % up to 5% were used in respective production flasks and productivity was checked after every 24 hours using pullulanase assay.

Effect of incubation time on pullulanase production: It has been observed that the culture shows maximum activity at particular time of incubation. The production flask was incubated up to drop in activity and pullulanase activity was measured after every 24 hours.

Effect of Temperature on Pullulanase production: Temperature plays a critical role in production of enzyme as culture shows maximum efficacy for enzyme production at particular temperature. In the present study, production flask was incubated at various temperatures at 30ºC, 35ºC, 37ºC, 40ºC, 45ºC and 50ºC. Pullulanase activity was measured after every 24 hours.

Effect of pH on pullulanase production: Production medium was prepared with varying pH (4-9) in the interval of 1. The flask was incubated for optimized time and at optimized temperature. After every 24 hours, pullulanase activity was observed.

Effect of agitation rate (RPM) on pullulanase production: Agitation rate in submerged fermentation maintains the rheology of medium which is necessary to provide proper dissolved oxygen and even distribution of medium for optimum enzyme production. But high agitation rate results in damage to cell structure and yield of secondary metabolites decreases[11].Production flasks were incubated at different RPM ranging from 100 RPM to 180 RPM at the interval of 20 RPM and enzyme activity was measured after every 24 hours.

Optimization of chemical parameters
Effect of Crude substrate on pullulanase production: 0.5% of different crude carbon sources like corn flour, sago starch, rice bran, red gram and wheat bran were used as substrate for pullulanase production. After every 24 hours, enzyme was extracted using centrifugation and assay was carried out.

Effect of different Carbon source on pullulanase production: To check the effect of different carbon sources, 0.5% pullulan was replaced by glucose, sucrose, starch and maltose in the basal medium and enzyme activity was measured after every 24 hours.

Effect of different concentration of pullulan and starch on pullulanase production: The optimum concentration of carbon source is necessary in production medium to achieve optimum growth and productivity. In the present study, varying concentrations of pullulan and starch ranging from 0.1% to 1% with interval of 0.2% were studied and enzyme activity was measured up to 72 hours.

Effect of Nitrogen source on pullulanase production: In the basal medium, peptone was used as nitrogen source which was replaced by yeast extract, tryptone, casein, ammonium acetate, ammonium sulphate and sodium nitrate affecting the growth of bacteria by its crucial need to full the metabolic pathways[18]. To check effect of nitrogen source on pullulanase production, enzyme was extracted after every 24 hours and activity was measured.

Effect of different concentration of Peptone on pullulanase production: The optimum concentration of peptone is necessary in production medium that improves and boosts growth of bacteria by increasing the production of essential enzymes[18]. The effect of different peptone concentration was tested by adding 0.4 to 2 % peptone with interval of 0.4% in production medium. In the present study, varying concentrations of peptone from 0.4 to 2 % with interval of 0.4% were checked with the combination of pullulan and starch.

Effect of different metal ions on pullulanase production: The effect of different ions (Ca^{+2}, Cu^{+2}, Co^{+2}, Mn^{+2}, Mg^{+2}, Zn^{+2}, Ni^{+2}, Fe^{+2}) on the pullulanase production was studied at 0.2 mM concentration. These ions were included in the production medium as the $CaCl_2$, $CuCl_2$, $CoCl_2$, $MnCl_2$, $MgCl_2$, $ZnSO_4$, $NiSO_4$ and $FeSO_4$ salts and after every 24 hours, pullulanase activity was measured.

Results and Discussion

Bacterial culture: Total 57 bacterial strains were isolated from enriched media and screened for the pullulanase producing capabilities at 37º C and pH 7.5 on plates containing pullulan. Pullulan-degrading microorganisms were identified by the presence of clear zones (haloes) around colonies, after immersing the culture in ethanol (99 % (v/v)) for 3 h[2,26]. After primary screening, 8 bacterial strains were screened which were selected for secondary screening. In secondary screening, NP 7 bacterial strain gave 33.33 U/ml maximum pullulanase activity among other cultures based on which NP 7 bacterial strain was selected for future work and microscopic observation of culture is shown in fig. 1.

Optimization of physical parameters

Effect of inoculum size: In the present study, production medium inoculated with 3 % inoculum shows maximum productivity at 24 hours which was similar to *Bacillus cereus* FDTA 13[19]. Above 3% inoculum activity starts decreasing as rheology of media is not maintained due to overgrowth of culture (Graph 1). An inoculum size of less than 3% was found to be insufficient for growth and enzyme production.

Effect of incubation time: Study of incubation time is necessary for any experiment as it gives an idea of length of entire fermentation process, moreover, it gave an idea of actual time when culture is producing maximum enzyme. In the submerged fermentation, NP 7 shows 41.34 U/ml pullulanase activities at 48 hours which was maximum (Graph 2). *Bacillus cereus* also shows maximum extracellular pulullanase production at 48 h with 0.55U/ml activity[25].

Effect of incubation Temperature: In the present experiment, productivity of pullulanase was checked in range of temperature between 30ºC to 50ºC.The maximum yield of pullulanase was observed at 45ºC (35.50 U/ml) (Graph 3). In contrast, *Bacillus* sp. and *Klebsiella pneumonia* were reported to exhibit optimum pullulanase production at 50ºC temperature by Waleed et al[25] and Abdul-Hadi et al[1]. Further increase in temperature suppressed the growth of bacteria and consequently enzyme production also gets inhibited.

Effect of pH: NP 7 bacterial culture shows maximum pullulanase enzyme production at 7 pH (Graph 4). Different organisms have different pH optima and decrease and increase in pH result in poor microbial growth. Enzyme production stimulates at neutral pH and also increases the cell growth. Neutral pH was also found to be optimal for pullulanase production in *Bacillus safensis*[21].

Effect of agitation rate: Present study shows that 120 RPM was favorable for maximum yield of pullulanase. On further increase of RPM, production of pullulanase was decreased (Graph 5). For proper balance of mixing of nutrients and their physical availability to the organism, agitation is necessary. The uptake of nutrients from the outer environment may be hindered, as too little time is available for contact with substrates at higher agitation rate. This may lead to reduced uptake of substrates and other nutrients resulting in poor growth and production of enzymes. For the production of amylopullulanase from *Geobacillus thermoleovorans*, agitation rates in the range of 100 to 250 rpm were checked for maximum production[3,20].

Optimization of chemical parameters

Effect of crude carbon substrate: In the present study, different crude substrate such as corn flour, sago starch, rice bran, red gram and wheat bran were studied with 0.5% liquid production medium. The result reveals that the maximum productivity (123.00 U/ml) was obtained in control production (Graph 6) after 48 hours.

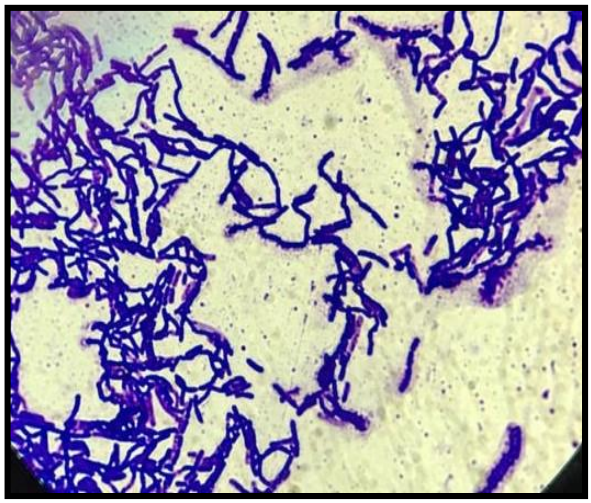
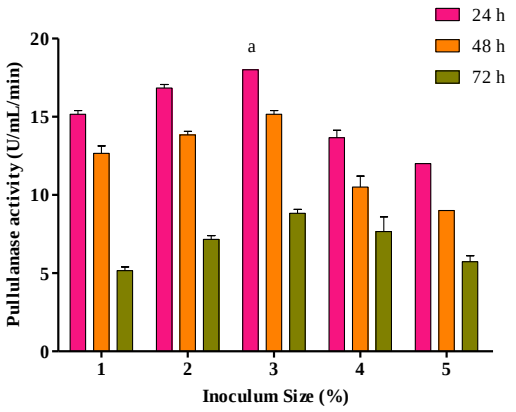
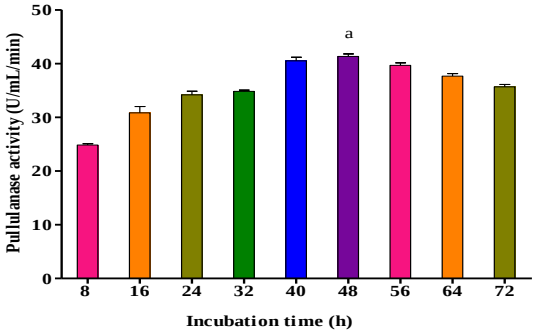


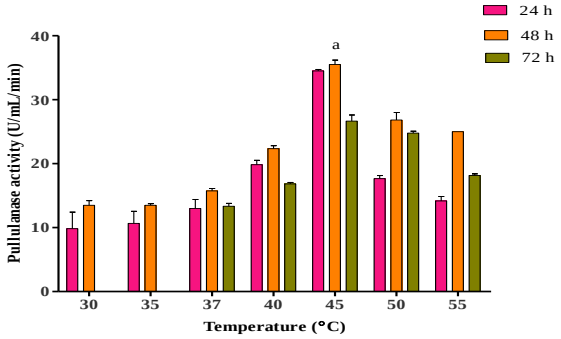
Figure 1: Microscopic observation of NP 7 strain



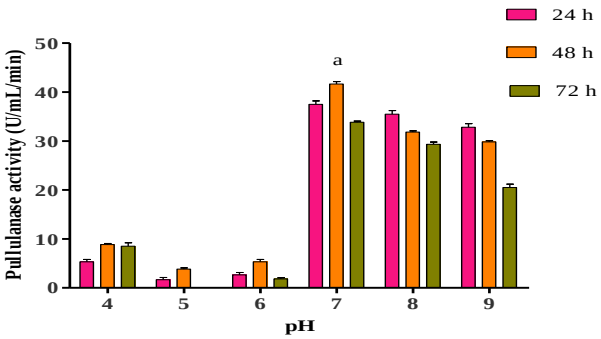
Graph 1: Effect of Inoculum Size: p<0.0001, p value summary based on Tukey's multiple comparison tests.
[a] highly significant at a time interval of 24 hrs



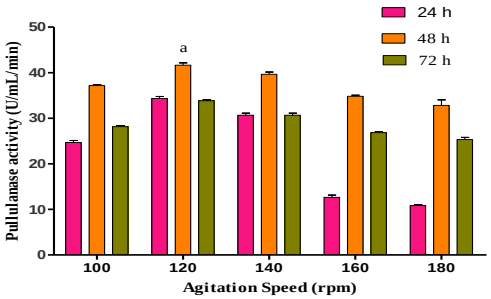
Graph 2: Effect of Incubation time: p<0.0001, p value summary based on Tukey's multiple comparison tests.
[a] highly significant at a time interval of 24 hrs.



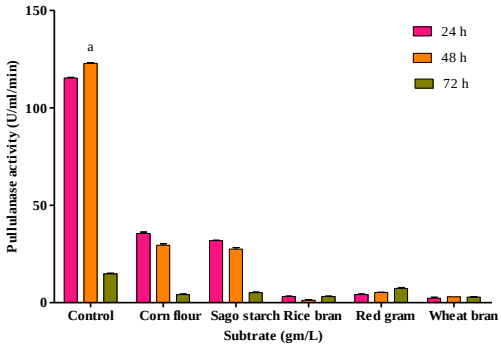
Graph 3: Effect of Temperature: p<0.0001, p value summary based on Tukey's multiple comparison tests.
[a] highly significant at a time interval of 24 hrs



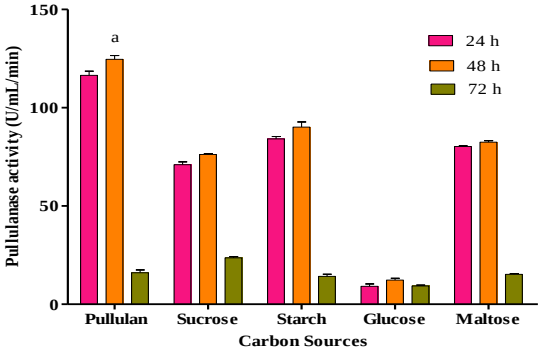
Graph 4: Effect of pH: p<0.0001, p value summary based on Tukey's multiple comparison tests.
[a] highly significant at a time interval of 24 hrs



Graph 5: Effect of agitation rate: p<0.0001, p value summary based on Tukey's multiple comparison tests. [a] highly significant at a time interval of 24 hrs.



Graph 6: Effect of crude substrate: p<0.0001, p value summary based on Tukey's multiple comparison tests. [a] highly significant at a time interval of 24 hrs

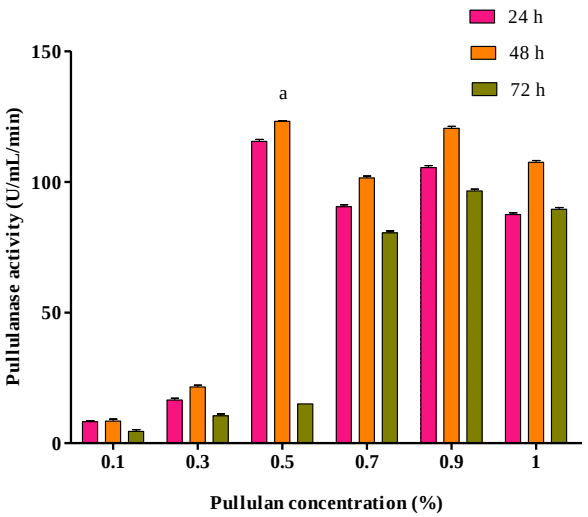


Graph 7: Effect of different carbon sources: p<0.0001, p value summary based on Tukey's multiple comparison tests. [a] highly significant at a time interval of 24 hrs

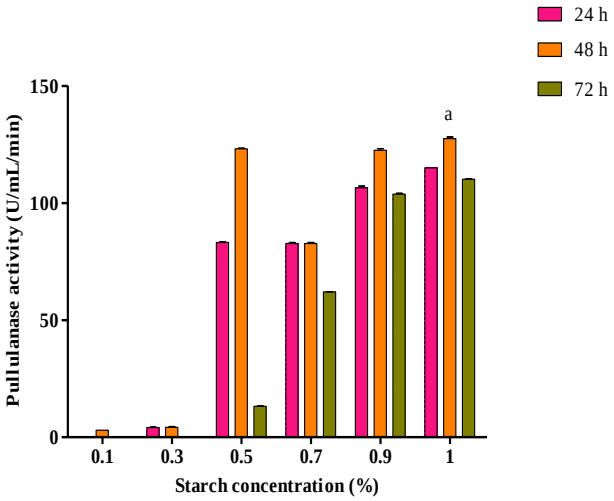
Effect of different Carbon source: In present study with NP 7, among different carbon sources, 0.5% starch gave 127.50 U/ml enzyme activities and pullulan gave 125.00 U/ml activities. Other carbon sources do not show any significant increase in enzyme activity (Graph 7). Maximum production of pullulanase by *Bacillus cereus* FDTA 13 was observed with soluble starch which increased the yield 1.8-fold as compared to control. For production of pullulanase, soluble starch is a good source of carbon because it can induce its release in case of cell associated pullulanase[19].

Effect of different concentrations of pullulan and starch in production medium: Different concentrations (0.1 to 1.0%) of pullulan and starch were studied and it was found that 0.5% pullulan gave maximum productivity of 123.00 U/ml (Graph 8) and 1% starch gave maximum productivity of 127.50 U/ml (Graph 9) which is similar. So, 1% starch can be used further for pullulanase production. Among the carbon sources, pullulan and starch were found to support pullulanase production. *B. cereus* showed high enzyme yield (1.16 U/ml) when pullulan was used as carbon source. Similar observation was noticed for an enhancement of pullulanase synthesis by pullulan in *B. cereus* FDTA-13 [19] and in *B. halodurans*[4,25].

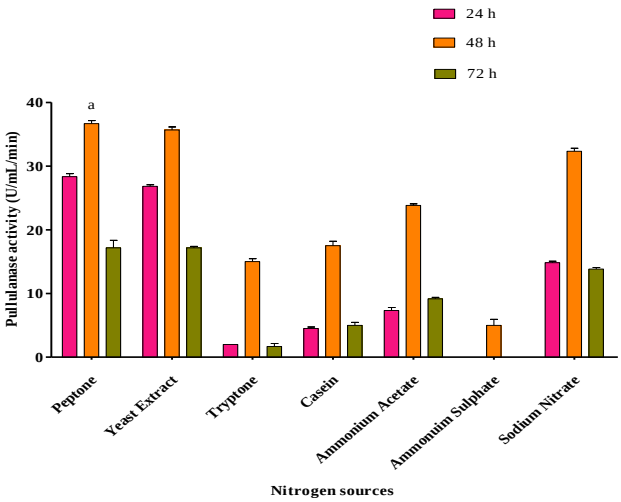
Effect of different nitrogen source: In present study with NP 7, it was observed that peptone showed highest productivity of pullulanase enzyme compared to other nitrogen sources (graph 10). Peptone can be easily degraded and absorbed because of low molecular weight[15]. Among the different nitrogen sources tested, peptone was more preferred for extracellular pullulanase production. Similar observation was noticed for *Clostridium thermosulfurogenes* SV9[4].



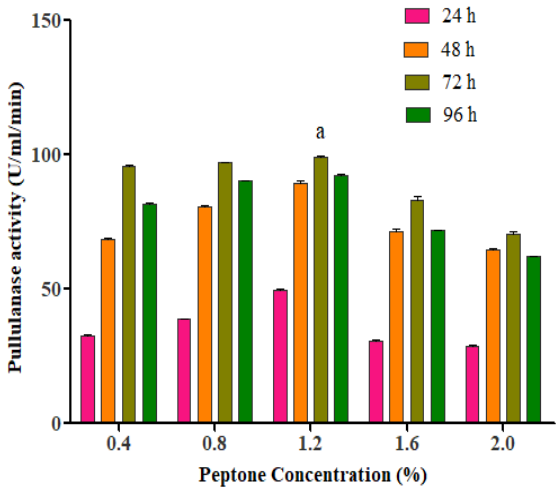
Graph 8: Effect of different Pullulan concentration: p<0.0001, p value summary based on Tukey's multiple comparison tests. [a] highly significant at a time interval of 24 hrs



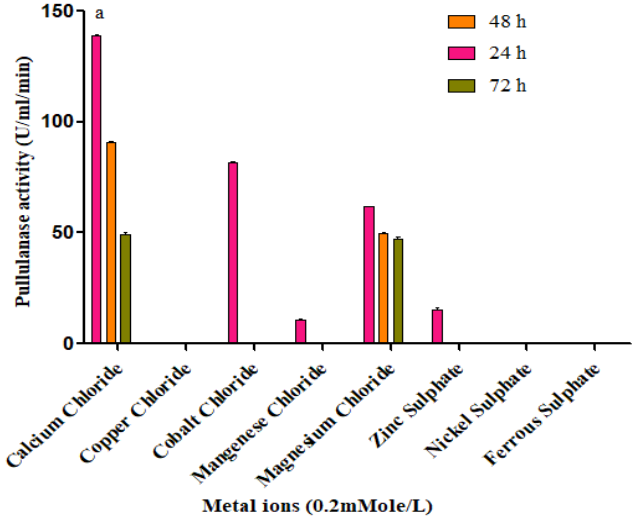
Graph 9: Effect of different Starch concentration: p<0.0001, p value summary based on Tukey's multiple comparison tests. [a] highly significant at a time interval of 24 hrs



Graph 10: Effect of different nitrogen sources: p<0.0001, p value summary based on Tukey's multiple comparison tests. [a] highly significant at a time interval of 24 hrs



Graph 11: Effect of different Peptone Concentration: p<0.0001, p value summary based on Tukey's multiple comparison tests. [a] highly significant at a time interval of 24 hrs.



Graph 12: Effect of different mineral salts: p<0.0001, p value summary based on Tukey's multiple comparison tests. [a] highly significant at a time interval of 24 hrs

Effect of different concentrations of nitrogen source in production medium: Different concentrations of peptone ranging from 0.4 to 2.0% (interval of 0.4%) were studied and it was found that 1.2% peptone gave maximum pullulanase activity (Graph 11) which is same as original production medium which was similar to *Bacillus halodurans* and *Bacillus licheniformis* for production of extracellular pullalanase[18].

Effect of different metal ions: Pullulanase is known to be metalloenzyme. Supplementation of salts of certain metal ions provided good growth of bacteria and thereby better enzyme production**.** Present medium contains metal ions. Mineral salts played an indirect role in production of enzyme. From the result, it was found that 0.2 mM/L concentration of $CaCl_2$ showed maximum activity of enzyme than the control which contains $MgSO_4$ (Graph 12) which was similar to thermophilic *Bacillus strain* 3183. For stabilization and maintenance of pullulanase activity, Ca^{+2} might be required[20].

Therefore, $CaCl_2$ acts as activator for enzyme production and $NiSO_4$ and $FeSO_4$ act as inhibitors. $FeSO_4$ and $BaCl_2$ have the highest inhibition effect on pullulanase production by *B. cereus* KKSJ 1981[15]. Nair et al[19] observed similar results for pullulanse production from *Bacillus* sp. and *B. cereus* respectively.

Conclusion

NP 7 has shown 4 times increase in productivity of pullulanase after optimizing physical and chemical parameters. After optimization of physical parameters, NP 7 gave maximum pullulanase production using 3% inoculum at 45ºC temperature and at 7 pH after 48 hours of incubation in submerged condition with 120 RPM. Starch, peptone and $CaCl_2$ maximize production of enzyme at the concentrations of 1%, 1.2 % and 0.2 mM/L respectively. Optimized

parameter can be used to set up production of pullulanase at pilot or large scale production.

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