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Screening, Purification and Characterization of Pullulanase from Soil Bacteria and Enhancement of Its Stability by Immobilization and Nanoforms

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Abstract
Pullulanase is an extracellular carbohydrase responsible for the hydrolysis of pullulan and amylopectin to produce maltotriose. The product maltotriose is used in detergent industry, bakery industry and in the production of biotechnological products. In the present investigation pullulanase was purified from bacillus species isolated from soil sample. The pullulanase was purified by salt precipitation (70%) followed by an-ion exchange chromatography (DEAE- Cellulose) and gel filtration (Sephadex G75) with final yield of 75% and purification fold 16.14. The molecular mass of pullulanase enzyme was 110 kDa as estimated by SDS-PAGE. The enzyme activity of purified sample was found to be 1294 IU and specific activity 8271.8. This study also aimed to determine the stability of immobilized pullulanase and showed high stability using Cu Nanoparticle.
Key words: - *Pullulanase, Gel filtration. SDS-PAGE, Nanoparticle.*

Introduction

Pullulanase is an extracellular carbohydrase which debranches the extracellular yeast polysaccharide and pullulan. Pullulanase is responsible for the hydrolysis of pullulan and amylopectin to produce maltotrios.

Pullulan+H_2O $\xrightarrow{\text{Pullulanase}}$ Maltotriose

Pullulanase was first isolated from mesophilic organism *Klebsiella pneumoniae* by Bender and Wallenfels in 1961 [28]. Microbial pullulanase attracts more interest because of its specific action on α- 1, 6 linkages in pullulan, a linear α- glucan consisting essentially of maltotriosyl units connected by 1, 6α-bonds.

Pullulanase is of great significance due to its wide area of potential application. It is a very potent enzyme for degradation of starch to glucose and maltose. It has been reported that Pullulanase enzyme is used on a large scale in glucose and maltose syrup industries. It is widely used in industries in the Saccharification of Starch. It converts starch into glucose and maltose which are used in the production of glucose syrup more efficiently[28]. Pullulanase is used in detergent industry (Van., and Willem., 1990), baking industry and for the production of cyclodextrins which in turn is used in the production of Biotechnological products and low calorie beer [14].

A number of pullulanase have been purified and characterised from different bacterial sources. Pullulanase type I has been characterised from mesophilic bacteria such as *Aerobacter aerogenes* [27], *Bacillus acidopullulyticus, Klebsiella pneumonia* and *Streptomyces sp.* Moderate thermophilic gram positive bacteria such as *Bacillus flavocaldarius, Bacillus thermoleovorans, Clostridium sp,* and *Thermos caldophilus* also have ability to secrete pullulanase type I. Pullulanase type I from hyperthermophilic bacterium *Fervidobacterium pennavorans*, has also been reported.

In the present investigation pullulanase production was screened from soil bacteria at different flour mills in Bangalore and the moicroorganism was identified by microscopic and biochemical tests. The enzyme was then purified, characterized and its stability was checked by immobilization and nanoforms.

Materials and Methodology

Screening, Isolation and identification of microorganism for the production of pullulanase enzyme

Collection of soil sample: Soil sample from different flour mills was collected and 1g of sample was weighed and a suspension was prepared using 10ml of saline. It was allowed to settle down and the clear top layer in the tube was used as inoculums.

Isolation of organism by using specific media: The pullulanase media was sterilized and 500µl of the inoculum was inoculated through pour plate method. The plates were Incubated at 37ºC for two days and observed for the growth of microorganism in each plate. The colonies were marked; pure cultures of those isolated colonies were maintained.

Screening for the production of pullulanase

Enzyme assay for crude enzyme sample: Pullulan broth was prepared and auto claved, the test organism was inoculated into the broth and incubated at 37ºC. After 24 hrs, 1ml of broth was taken and centrifuge at 10,000 rpm for 10 min. The supernatant was used as crude enzyme source. 0.35ml of phosphate buffer, 0.05ml of 2% pullulan was taken in the test tubes, pre-incubated at 37ºC for 10min. 0.1ml of crude enzyme was added and incubated at 37ºC for 10 min. 0.5ml of copper reagent was added to stop the reaction, incubated in boiling water bath for 60 min. 0.5ml of arseno-molybdate reagent and 11ml of distilled water was added. Blank was processed in the same way without substrate. Absorbance was measured at 546nm and enzyme activity was calculated [31].

Identification of microorganisms by morphological and biochemical test: The colony characteristics of the test organism was observed and recorded.

Gram staining: A thin smear of the pure culture was made on a clean glass slide, heat fixed. Crystal violet was added, left for 1min followed by water wash, added grams iodine for 1min and washed, 70% alcohol for 30 sec, washed and added a counter stain saffrainin for 30 sec and observed under oil immersion.

Methyl red – Voges proskauer: MRVP broth was autoclaved and about 10ml of broth was taken in four test tubes. Labelled as MR control, MR test, VP control, VP test. The test organism was inoculated into the MR test and VP test tubes, all the tubes were incubated at 37ºC for 24 – 48 hrs. After the incubation time methyl red solution was added to the MR test and control tubes. For VP, Barrets reagent I (40% KOH) and Barrets reagent II (α-naphthol in 95ml of 95% alcohol) was added and observed for the results.

Catalase test: A thin smear of the pure culture was made on a clean glass slide and a drop of H_2O_2 was added to the culture and observed for the result.

Indole test: 1% Tryptone broth was prepared and 10ml of broth was taken in two tubes. Labelled one as control and one as test. Inoculate the tube (test) with the test organism and incubated at 37ºC for 24 – 48 hrs. After incubation few drops of kovac's reagent was added and observed for the results.

Citrate utilization: The media was prepared, autoclaved and two slants were prepared out of which one was labelled as control and another as test. The tube (test) was inoculated with the test organism and incubated for 2 days to observe the results.

Triple Sugar Iron Agar: TSI agar was prepared. After sterilization of the media two slants were prepared and labelled as control and test. The test organism was inoculated only into the tube labelled as test by streaking and control was left uninoculated. Incubated the tubes at 37ºC for 2 days and observed for the colour change from red to yellow.

Gelatin hydrolysis: Gelatin hydrolysis media was prepared, autoclaved and 10ml of it was taken in two test tubes and labelled. The test organism was inoculated into the tube labelled as test by stabbing

and the control was left uninoculated. Incubated the tubes at 37ºC for 2 days and observed for the results.

Casein hydrolysis: The Casein hydrolysis broth was subjected to pasteurization at 75ºC for 35min. 0.9g of agar was dissolved in 100ml distilled water and auto claved in separate beaker. The pasteurized milk powder and agar solution was mixed. The media was poured onto two petriplates –control and test.The test organism was inoculated by zigzag streaking and the control was left uninoculated. Incubated the petriplate at 37ºC for 2 days and observed for the result.

Lipase reductase: Lipase reductase media was prepared, autoclaved and poured onto two petriplates and was labelled as control and test. The control petriplate was left uninoculated and test was inoculated with the test organism. Incubated the plates at 37ºC for 2 days and observed for the results.

Urease: Urease media was prepared, the pH was set to 6.8 and 0.04g of phenol red and 0.6g agar was added and autoclaved. 10 ml of distilled water was autoclaved and membrane sterilized and UV sterilized. 4g of UV sterilized urea was added to 10ml sterile water. Both the solution were mixed. Slants were prepared out of this media and labelled as control and test .Control was left uninoculated and test was inoculated with the test organism. These tubes were incubated at 37ºC for 2 days.

Nitrate reductase: Nitrate broth was prepared, autoclaved and 10ml of broth was taken in two test tubes. Labelled as control and test. Test was inoculated with the organism. Incubated the tubes at 37ºC for 24 -48hrs. 1-amino-2-naphthol-4-sulphonic acid was added dropwise and observed for red colour. For confirmation zinc dust was added.

H_2S production: SIM agar was prepared, autoclaved and 10ml was poured onto two tubes – control and test. The culture was inoculated to the tube (test) by stabbing and the control is left uninoculated. Incubated the tubes at 37ºC for 24-48 hrs.

Carbohydrate fermentation: 250 ml of Carbohydrate fermentation broth was taken in four different beakers and labelled as glucose, lactose, sucrose, and mannitol. 5g of the respective sugars and 0.0045g of phenol red was added to each beaker.

10ml of each fermentation broth was transferred on to two test tubes and Durham's tube wass added with care. After autoclaving, tubes were labelled as control and test in each of the four sugar fermentation media prepared. Only the tubes labelled as test was inoculated with the test organism. Incubated the tubes at 37ºC for 2 days. [1]

Purification of pullulanase enzyme:

Salt precipitation: The 48 hrs incubated broth was centrifuged at 10,000 rpm for 10min. The supernatant was taken, stirred on the magnetic stirrer, ammonium sulphate was added until saturation. It was incubated overnight and centrifuged again at 10,000rpm for 10 min. The pellet was collected and dissolved in 10 mM, 10ml Tris HCl [11].

Dialysis: Primarily the dialysis bag was activated by placing in 100ml boiling water for 20min. 2% $NaHCO_3$ was added in pinch to the boiling water in order to unclose the pores in the dialysis bag and kept at boiling for 15 min. the dialysis bag was transferred onto fresh boiling water to remove the excess $NaHCO_3$. It was allowed to cool at room temperature. The activated bag was tied at one end and using micropipette, the enzyme dissolved in Tris HCL was added slowly into the dialysis bag. A beaker was filled with distilled water and the dialysis bag with the enzyme sample was immersed in it, incubated in refrigerator overnight. Then it was stirred on a magnetic stirrer for 2hrs with regular change of water after every 1/2hr. Tthe sample in the dialysis bag was then transferred to the centrifuge tube and centrifuged at 10,000rpm for 10min, the supernatant was taken for further processing [36].

Ion exchange chromatography: The column was cleaned with methanol, sonicated for ½ hr., washed with distilled water. The column was fixed on to the stand and filled with DEAE cellulose resign, which is an anionic exchange resign. Different elution's were prepared by mixing tris HCl and NaCl in

different concentration (250/250,250/500, 250/700, 250/1000, 250/1250, 250/1500, 250/1750). The elution $E_1$was layered over the resign and eluted out, this elution was discarded. Then the sample was layered over the resign and eluted out using the 2nd elution, the eluted sample was collected and processed. Now 3rd elution was layered and eluted sample was collected and processed. The same was repeated with the remaining elution's E_4,E_5,E_6,E_7 and the eluted samples were collected and enzyme assay was performed for each of the eluted sample and protein concentration was estimated [15].

Gel filtration: Gel filtration was carried out using G_{75}resign in the column for filtration. 20ml phosphate buffer of pH 7.5 was prepared. The column was3/4th filled with G_{75}resign then the eluted sample of ion exchange showing high activity was layered over the resign and the remaining space in the column was filled with phosphate buffer and the purified sample was collected by opening the outlet valve in 20 eppendoff tubes. Protein eastimation and enzyme assay was performed for all the samples collected [21].

SDS PAGE: Preparation of separating gel: 3ml of stock solution (35% acrylamide and 0.8% of bisacrylamide) was taken in a test tube, to that 2.6ml of separating buffer and 1.5ml of distilled water was added and sonicated for 10min.To this 100µl SDS, 100µl APS, 20µl TEMED was mixed and poured onto the gel cassette till ¾.

Preparation of stacking gel: 786µl of stock solution, 3ml of distilled water, 1 ml of stacking buffer was taken in a test tube and sonicated for 5min. To this 100µl SDS, 100µl of APS, 20µl of TEMED was mixed and layered over the separating gel and the comb was inserted immediately to create wells. The gel was allowed to solidify for about 20min, then the spacer at the base was removed and fixed on to the SDS PAGE setup. Samples and marker were loaded in their respective well, connected to the power supply and allowed to run at 50V for an hour then increased to 100V for 4hrs. The power supply was switched off and the gel was processed by placing it in staining solution for overnight and then transferred to destaining solution. The destaining solution was changed every 2 hours till the bands were clearly visible[20].

Enzyme kinetics of purified enzyme:

Effect of pH on enzyme activity: Using buffers of different pH the enzyme assay was performed and the optimum pH at which the enzyme activity is maximum was found out.

Effect of Temperature on enzyme activity: The regular protocol of enzyme assay was followed and the tube were incubated at different temperature and optimum temperature at which the enzyme activity is maximum was recorded.

Effect of Incubation time on enzyme activity: : The regular protocol of enzyme assay was followed and the tubes were incubated at different time duration (5, 10, 15, 20, 25min). The tube showing highest activity at an optrimum incubation time was recorded.

Effect of Substrate concentration on enzyme activity: Different aliquots of substrate 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07ml were taken in the test tubes, the enzyme assay was performed. The tube showing maximum absorbance at 546nm was reported as optimum substrate concentration[13].

Stability check of pullulanase enzyme by various immobilization methods

Entrapment by alginate method: 3% sodium alginate was prepared and 5ml of it was mixed with 0.5ml of enzyme, beads were made by droping it drop wise in 0.2M calcium chloride. The enzyme activity of immobilized enzyme was calculated by performing enzyme assay. Blank was processed using alginate beads without enzyme. The immobilized enzyme was stored for about seven days and the enzyme assay was repeated, to check the stability of immobilized enzymes [12].

Entrapment by polyacrylamide gel: 15% polyacrylamide gel was prepared by dissolving 7.5g acrylamide, 0.5g bisacrylamide and 50mg ammonium per sulphate in 25ml of phosphate buffer (pH-6.8). To 2.5ml of polyacrylamide gel 1ml of enzyme was added, layered it on the petriplate and was cut into pieces. The enzyme assay was performed using these pieces. Blank was

processed using the polyacrylamide gel without enzyme. The acrylamide immobilized enzyme was preserved for a week and checked for its stability [37].

Entrapment by polyurethane: 2g of urethane prepolymer was cooled at 4ºC, mixed with 2ml chilled phosphate buffer (pH-5) and 1ml of enzyme and incubated at room temperature. After polymerization and formation of foam the gel was rinsed with the buffer. The gel was cut into small pieces and used as immobilized enzyme samples and the enzyme assay was performed. The immobilized enzyme was preserved for a week and enzyme assay was performed to check the stability [18].

Stability check of Pullulanase enzyme by various nanoforms:

- Nanoparticles of Cu with concentration of 2.1µg/ml and 14.7µg/ml were prepared, incubated with the enzyme for about 5hrs with constant stirring. The enzyme assay was then performed using nanoparticles immobilized enzyme sample to find out the enzyme activity and the same sample was stored for a week. On the seventh day the enzyme assay was repeated with these nano-enzymes inorder to check the stability of the nano-enzymes.
- Nanoparticles of Zn with concentration of 2.1µg/ml and 14.7µg/ml were prepared, incubated with the enzyme for about 5hrs with constant stirring. . The enzyme assay was then performed using nanoparticles immobilized enzyme sample to find out the enzyme activity and the same sample was stored for a week. On the seventh day the enzyme assay was repeated with these nano-enzymes in order to check the stability of the nano-enzymes.
- Nanoparticles of Ag with concentration of 2.1µg/ml, 14.7µg/ml were prepared, incubated with the enzyme for about 5hrs with constant stirring. The enzyme assay was then performed using nanoparticles immobilized enzyme sample to find out the enzyme activity and the same sample was stored for a week. On the seventh day the enzyme assay was repeated with these nano-enzymes inorder to check the stability of the nano-enzymes [24].

Results

Isolation, screening and identification of pullulanase producing bacteria: The bacteria was isolated from the soil and screened for pullulanase production by growing it in pullulanase specific media. Based on biochemical tests, morphological microscopic observation the organism was found to be bacillus sp.

Biochemical tests	Result
Catalase	+
Carbohydrate fermentation	+
Indole	-
Methyl red	-
Voges proskauer	-
Citrate utilization	+
H_2S production	-
Starch hydrolysis	-
Casein hydrolysis	+
Lipase	-
TSI	+
Urease	-
Nitrate	-
Gelatin hydrolysis	+
Oxidase	-

Biochemical tests:

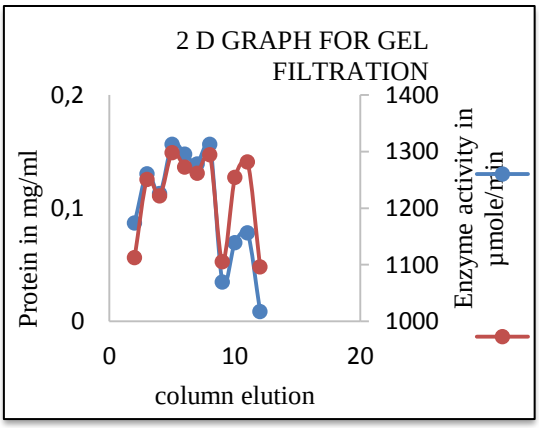
By analysing all the morphological, microscopic and biochemical tests showing positive results and on comparing the results with that of bergey's manual, the organism was found to be Bacillus species.

Enzyme production and purification: Pullulanase is an extracellular enzyme and therefore will be present in the broth. Hence the broth was used as

crude enzyme sample and the assay was performed, the positive results of which confirmed the presence of pullulanase enzyme. This crude enzyme sample was purified by series of purification steps like salt precipitation, dialysis, anion exchange, gel filtration and the purification fold increased after each purification step.

Table 1: Purification table showing purification fold and % recovery.

Sample	Enzyme activity	Protein	Specific activity	Purification fold (F)	Recovery %
Crude	970.9	1.8956	512.19	1	100
Salt precipitation	990.9	0.4521	2191.77	4.2792	97.9816
Dialysis	1210.9	0.2869	4220.63	8.2403	80.18
Ion exchange	1254.54	0.1913	6557.97	12.803	77.3909
Gel filtration	1294.54	0.1565	8271.82	16.1499	74.9996



Graph 1: Graph representing the amount of protein and enzyme activity present in each of the column elution's collected during the process of gel filtration.

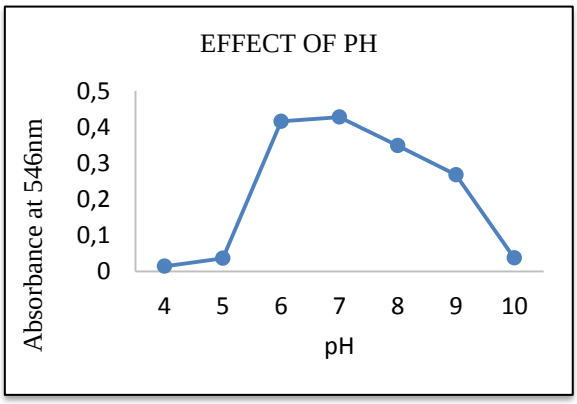
SDS PAGE

Fig 1: The purified pullulanase enzyme sample was subjected to SDS PAGE along with a MW marker and the molecular weight of Pullulanase was found to be 110 kDa.

Optimization of culture conditions for maximum pullulanase production: The organism cultured in pullulanase specific pullulan broth showed optimum production at a pH 6 -7, after which the enzyme production was found to be decrease as there was increase in the pH. The enzyme production was found to be high at a 37ºC temperature and a slow decrease in enzyme production was observed as the temperature increased.

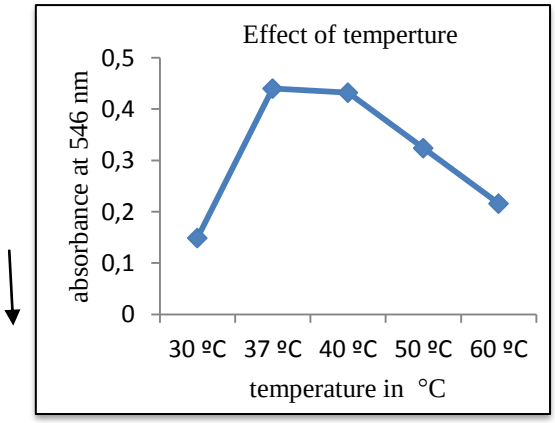
Pullulanase enzyme kinetics:

The optimum pH at which the enzyme activity was maximum was found to be pH-7.



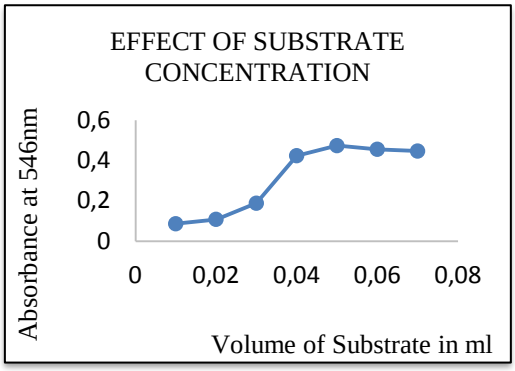
Graph 2: Graph showing maximum pullulanase activity at pH 7.

The optimum temperature at which pullulanase activity was maximum was found to be 37°C.



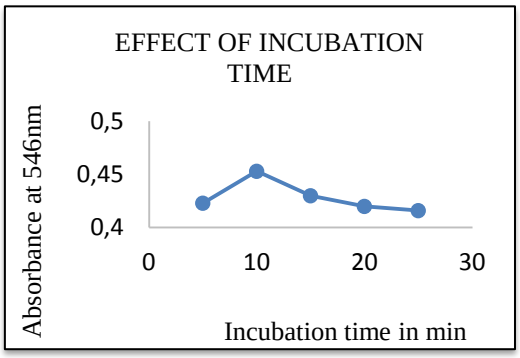
Graph 3 : Graph showing maximum pullulanase activity at 37°C.

The substrate concentration was found to be optimum at 0.05mL.



Graph 4 : Graph showing optimum pullulanase activity at 0.05mL substrate concentration.

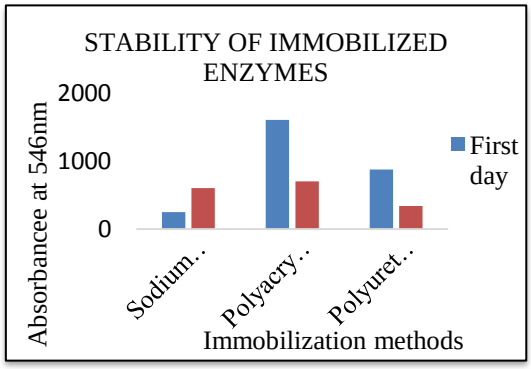
The optimum incubation time was found to be 10min.



Graph 5: Graph showing maximum pullulanase activity at 10min incubation time.

Stability check of pullulanase by various immobilization methods:

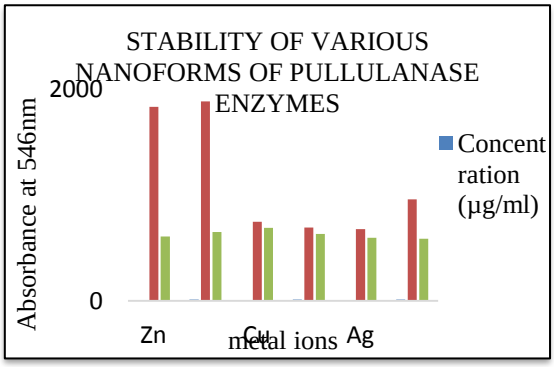
By comparing the activity of pullulanase immobilized by various methods it was found that the enzyme activity was maximum in polyacrylamide immobilized pullulanase sample. But when the stability of polyacrylamide immobilized pullulanase was checked, the stability seems to be decreased whereas the stability of alginate immobilized enzyme was found to be high.



Graph 6: Representation of stability of immobilized enzyme on its enzyme activity. High stability was found in sodium alginate immobilized enzyme sample.

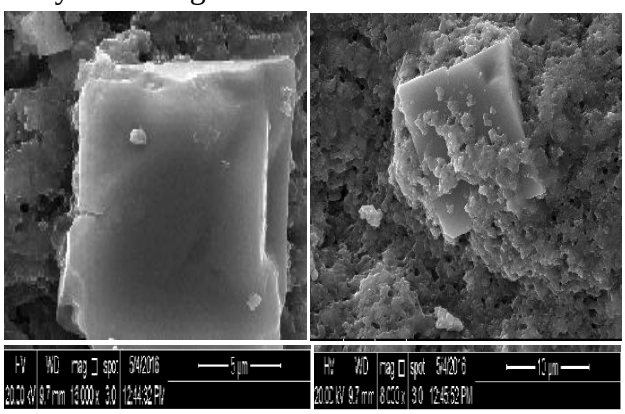
Stability check of pullulanase enzyme by various nanoforms:

The comparative study of enzyme activity of various nanoforms of pullulanase enzyme was found to be maximum in Zn nanoparticle immobilized enzyme but as far as stability was concerned Cu immobilized pullulanase sample was found to be quite stable.



Graph 7: Graphical representation of stability of nanoforms of pullulanase enzyme on its enzyme activity. High stability was observed in nanoform which is Cu immobilized.

Cu nanoparticle immobilized sample, which showed maximum activity was found to be 800nm when analyzed through SEM.



Pic 1: Scanning electron microscopic photograph of highly stable Cu nanoform of pullulanase enzyme.

DISCUSSION

The soil sample collected at different flour mills in Bangalore showed positive results for pullulanase production when plated on pullulanase specific media. The pure culture of micro organism was

screened for production of pullulanase enzyme. The substrate used was Pullulan. The Biochemical tests confirmed that Pullulanase was obtained from *Bacillus* species according to Bergey,s Manual . Maximum growth of bacteria and enzyme production was observed at 48 hours of incubation and it coincides with *Bacillus cereus* [38] where as 72 hours in extremophile bacterial species [28]. The optimum temperature for pullulanase was found to be at 37ºC in *Bacillus* species while it is 50ºC in *Bacillus halodurans* [5] whereas *Bacillus* sp. US149 [2], *Bacillus acidopullulyticus* [33] presented optimal temperature for a maximum pullulanase activity at 60°C.The organism showed optimum production at a pH 7, where as in *Bacillus halodurans it* was found to be 10 [5]. The molecular weight of Pullulanase from Bacillus species was found to be 110 KDa whereas pullulanase isolated so far has a MW of 98 KDa [28]. The purification fold of pullulanase enzyme in Salt precipitation was found to be 4.2792 and recovery percentage was 97.9816, in Dialysis the purification fold was 8.2403 and recovery percentage was 80.18, in Ion exchange the purification fold was 12.803 and recovery percentage was 77.3909.The purification fold of Gel filtration was 16.1499 and recovery percentage was 74.9996.The Purification fold of Pullulanase enzyme was found to be 16.1499 and recovery percentage 74.9996 from Bacillus species where as in Bacillus species KSM-1876 the purification fold is 83 and yield is 38%.The Optimization for the production of enzyme pullulanase with respect to pH was 7, temperature was 37ºC, incubation time was 10min, substrate concentration was 0.05 mL. The nanoparticles immobilized enzyme sample was prepared using 2.1µg and 14.7µg concentrations of metal ions such as zinc (2+), copper(2+) and silver(+1). Among these the copper immobilized sample showed high stability and its conformation remained undisturbed even after one week.

CONCLUSION

Microscopic observation and biochemical tests confirmed the production of pullulanase enzyme in *Bacillus species.* Final Purification fold of pullulanase enzyme was 16% and SDS PAGE showed that the molecular weight of pullulanase enzyme lies between 90-110Kda.Stability check was carried out by various immobilization methods, among them entrapment using sodium alginate gave best results. Immobilization of pullulanase using nanoparticle showed high stability using Cu. Hence from the present investigation immobilized pullulanase enzyme using nano forms can be used in the production of industrial products.

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