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ORIGINAL ARTICLE

Isolation, Screening and Characterization of Pullulanase producing *Bacillus subtilis*

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ABSTRACT
Agro-waste is produced in large quantities in a country like India and contains starch as one of its main ingredients. This waste material can be recycled to create new commodities that are marketable. Currently, the amylase enzyme is mostly used for saccharification of starch, although this process takes time. Pullulanase enzyme plays an economically significant role in the solution because it quickly saccharifies starch with a high yield of glucose or maltose. In the current investigation, bacteria, fungi and actinomycetes were isolated from different soil samples and food waste and Organisms were grown in enriched media supplemented with 1% starch, 1% glucose, and 1% sucrose added, respectively. A total of 57 bacteria, 10 fungi, and 5 actinomycetes were isolated and qualitatively screened using the agar plate screening method on pullulan containing media for the pullulanase enzyme. Eight bacterial isolates were selected based on maximum pullulan consumption zone, which were then quantitatively screened for the pullulanase enzyme in liquid medium using pullulan as a substrate. After 24 hours of incubation, bacterial culture (NP 7) produced the maximum pullulanase activity (33.33 U/mL). Furthermore, the strain that produced the highest amount of activity was characterized using biochemical and molecular techniques. The isolates were identified as Bacillus subtilis.
Keywords: *agro waste, amylase, Bacillus subtilis, enriched media, Pullulan, Pullulanase, Saccharification*

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INTRODUCTION

Pullulanase (EC 3.2.1.41) is known as debranching enzyme that targets the glucosidic bonds in pullulan and starch in particular [11]. Pullulanse is one of the industrial significant enzyme as it can hydrolyzes α 1,6-glycosidic bond of branched chains as well as the α 1,4- and α 1,6-glycosidic linkages of polysaccharides [3,5]. Pullulanase also has the ability to hydrolyze pullulan, into maltotriose units, making it a valuable enzyme for starch hydrolysis, which shows its application as an industrial debranching agent. [8,20,22]. As starch debranching enzymes, pullulanase have become more significant in current saccharification procedures, in the manufacturing of high purity glucose and fructose as well as maltose syrups in the starch processing sector [10, 13]. This happens when pullulanase is utilized in the saccharification process along with glucoamylase or a-amylase, as appropriate. In addition to starch hydrolysis, it is used in the bakery sector to remove staling and to produce branched cyclodextrin, which is utilized as a stabilizing agent, an order-removing agent, and a solubilizer for pharmaceuticals [17].

Pullulanases are a class of glycosyl hydrolases that are widely distributed in nature and are produced by a wide range of taxa, including animals, plants, and microorganisms [9, 11, 24]. Several pullulanases have been identified and characterized from bacterial sources such as *Aerobacter aerogenes, Bacillus acidopullulyticus, Klebsiella pneumonia Streptomyces sp., Bacillus flavocaldarius, Bacillus thermoleovorans, Clostridium sp, and Thermos caldophilus* [21].

The main purpose of this study is isolation of pullulanase producing microorganisms & identification of strain which produce maximum pullulanase.

MATERIAL AND METHODS

Collection of samples

Samples of food waste, baking trash, and soils were collected form several locations of Gujarat. Samples were bought in lab in sterile polybags (Figure 1).

Figure 1: collection of samples

Isolation of pullulanase producing microorganism

In three 250ml flasks, 5gm of sample was suspended in 50 ml of basal medium [(per litre): Nacl 1g, K2HPO4 5g, Yeast Extract 0.6g, MgSO4.7H2O 0.2g] supplemented with 1% of sucrose, glucose, and soluble starch respectively. Flasks were incubated at 30°C and 37°C different temperature for fungi and bacteria on a rotating shaker with a 120-rpm speed (144 hours). Bacterial strains were isolated from enriched soil samples on nutrient agar plates, while fungus cultures were isolated on potato dextrose agar. Nutrient agar plate and potato dextrose agar plate were incubated at 37^0C and 30^0C for bacteria, actinomycetes & fungi respectively.

Primary screening

The initial screening of isolates was carried out using a qualitative plate technique with 0.5% pullulan. Basal medium containing pullulan at concentrations of 0.5%, 1%, and 2% were used. Plates were incubated for pullulanase production of fungi with 30 ºC or bacteria with 37ºC and 45ºC. 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 at 4ºC [7].

Secondary screening

Crude enzyme preparation:

Bacterial culture grown on pullulan containing plates for 24 hours was inoculated in a 250 ml conical flask that contained 50 ml of basal broth supplemented with 0.5% pullulan. For 24 hours, the inoculated medium was incubated at 37 °C for bacteria while being rotated at 120 RPM.

Pullulanase Production:

At 660 nm, the turbidity was set to 2.0 in terms of absorbance, and each production flask were inoculated with 1% bacterial culture as an inoculant in 250 mL flasks containing 100 mL of pullulan medium, Flasks were incubated for 48 hours at 37 °C in rotatory shaker having 120 RPM. Samples were harvested after every 24 hours up to 48 hours period and centrifuged at 7500 rpm for 10 minutes at 4 °C. The pullulanase enzyme activity was assessed using the supernatant [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. 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, 19].

Identification of microorganisms by morphological and biochemical test

Gram staining:

The colony characteristics of isolates show pullulanase production was studied on plate. Gram staining of each culture was performed and morphology of cultures was observed under100X oil immersion lens.

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Biochemical Characterization of isolates:
According to the Bergey's manual of systematic bacteriology's biochemical parameters of isolates was performed using HiBacillusTM identification kit (Malonate, Voges Proskauer's, Citrate, ONPG, Nitrate reduction, Catalase, Arginine, Sucrose, Mannitol, Glucose, Arabinose, and Trehalose test) [4].

Molecular identification of isolates:
NP 7 culture is submitted to Gujarat State Biotechnology Research Centre (GBRC), Located in Gandhinagar, Gujarat, for 16S RNA sequencing using a DNA Sequencer (3500XL Genetic analyzers). The sequence was submitted to Gene Bank Database and the accession number of NP7 is OP615140.1. The sequence was analyzed for nucleotide sequence similarity using the Basic Local Alignment Search Tool (BLAST) (http: // blast.ncbi.nlm.nih.gov/blast.cgi) of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) to identify species. Mega XI software was used to create the phylogenetic tree using the maximum likelihood technique (version 11.0.7) [16].

RESULT AND DISCUSSION

Isolation of cultures:
From 7 different enriched samples for 144 h in basal medium containing 0.5% pullulan as a sole carbon source, a total of 57 bacteria, 5 actinomycetes and 10 fungal cultures were isolated. The isolated cultures of bacteria, actinomycetes & fungi were stored on Nutrient agar, Glucose asparagine agar and Potato dextrose agar slant respectively for future studies.

Primary screening of Pullulanase producer:
Qualitative assay for pullulanase production was carried out using selective plate method. All selected isolates were screened using Pullulan-containing media. From the addition of absolute alcohol, 8 bacterial strains from total 72 isolates yielded a positive result by showing the presence of clear zones (haloes) around colonies, after immersing the culture in ethanol (99 % (v/v)) for 3 h (Table 1)[7]. While promising results of pullulanase production was not found from Actinomycetes & Fungi culture (Figure 2).

Culture Code	Zone of clearance (mm)
NP1	1
NP2	0.5
NP3	0.3
NP4	1
NP5	1.8
NP6	2
NP7	4
NP8	3

Table 1: Showing zone of clearance in mm of different cultures

Figure 2: Pullulanase production by bacterial cultures

Pullulan is one of the most potent substrate for identification of pullulanase producer as five different types of Pullulanase enzymes can hydrolyze pullulan. These different enzymes may be recognized by the products they produce. Pullulan-degrading enzymes are among a class of glycosylhydrolases that are produced by a very diverse range of species and are widely present in nature. Many thermophilic and mesophilic bacteria are a significant source of the pullulanase enzymes [12, 22].

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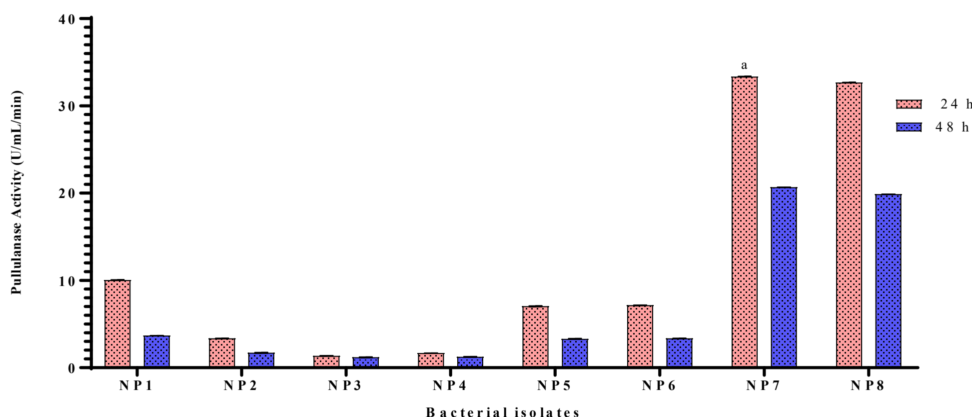
Similar to the present work, Waleed *et al.,* have identified 17 isolates for pullulanase enzyme by the observation of formation of transparent clear zone of pullulan hydrolysis around the colony using 0.5%, 1% and 2% Pullulan containing agar media [9]. Similar technique was used and Several *Bacillus* sp. was isolated from the soil and tested for the ability to produce pullulanase by cultivating it in media containing pullulan [15].

Secondary screening of Pullulanase producer:

After the initial primary screening, 8 best bacterial strains were selected for secondary screening where pullulanase production was checked in liquid culture using pullulanase assay. For the production of pullulanase, submerged cultivation method was used. The amount of enzyme activity was checked after every 24 hours up to 48 hours. The maximum enzyme activity was observed at 24 hours of pullulanase production. The maximum amount of pullulanase was produced by the bacterial isolate NP 7 (33.33 U/mL), NP8 also produced significant amount of pullulanase 32.66 U/ml. which was shown in graph 1 while NP1, NP6, NP5, NP2, NP4, and NP3 and produces 10.00,7.13, 7.00, 3.33,1.67, and 1.33 U/mL activity of pullulanases respectively.

Zhang *et al.*, Studied submerged fermentation method for the production of pullulanase using recombinant *Bacillus subtilis* and obtain enzyme activity of 102.75 U/ mL at 32 h [25]. In current study highest pullulan activity was observed at 24 h, While study in *Klebsilla aerogenes* NCIM 2239 show pullulanase activity peaked at 48 hours (78.62 U/mL) [18]. In the present study, it can be seen in graph 1, pullulanase production increased after the medium was incubated for 24 hours, reaching their peak at 24 h, with a concurrent decline in pullulanase production after 24 h.



Graph 1: Time course study for pullulanase production. ANOVA: p<0.0001, p value summary based on Tukey's multiple comparison tests = [a] highly significant at a time interval of 24 hrs.

Identification of microorganisms by morphological and biochemical test

Gram staining and Biochemical Characterization of isolated pullulanase producers:

On performing of gram staining, it was observed that isolated micro-organisms are gram positive. The result of Gram's reaction and biochemical characteristics are shown in Table 2,3 and figure 3.

Bacterial Code	Shape	Arrangement	Gram's Reaction
NP1	Medium Rod	Single, in chain	Gram +ve
NP2	Round	In cluster	Gram +ve
NP3	Short Rod	Single	Gram +ve
NP4	Short Rod	Single	Gram +ve
NP5	Rod	Single, in chain	Gram +ve
NP6	Rod	Single, in chain	Gram +ve
NP7	Rod	Single, in chain	Gram +ve
NP8	Rod	Single, in chain	Gram +ve

Table 2: Summary of Gram's staining for isolated bacterial strains

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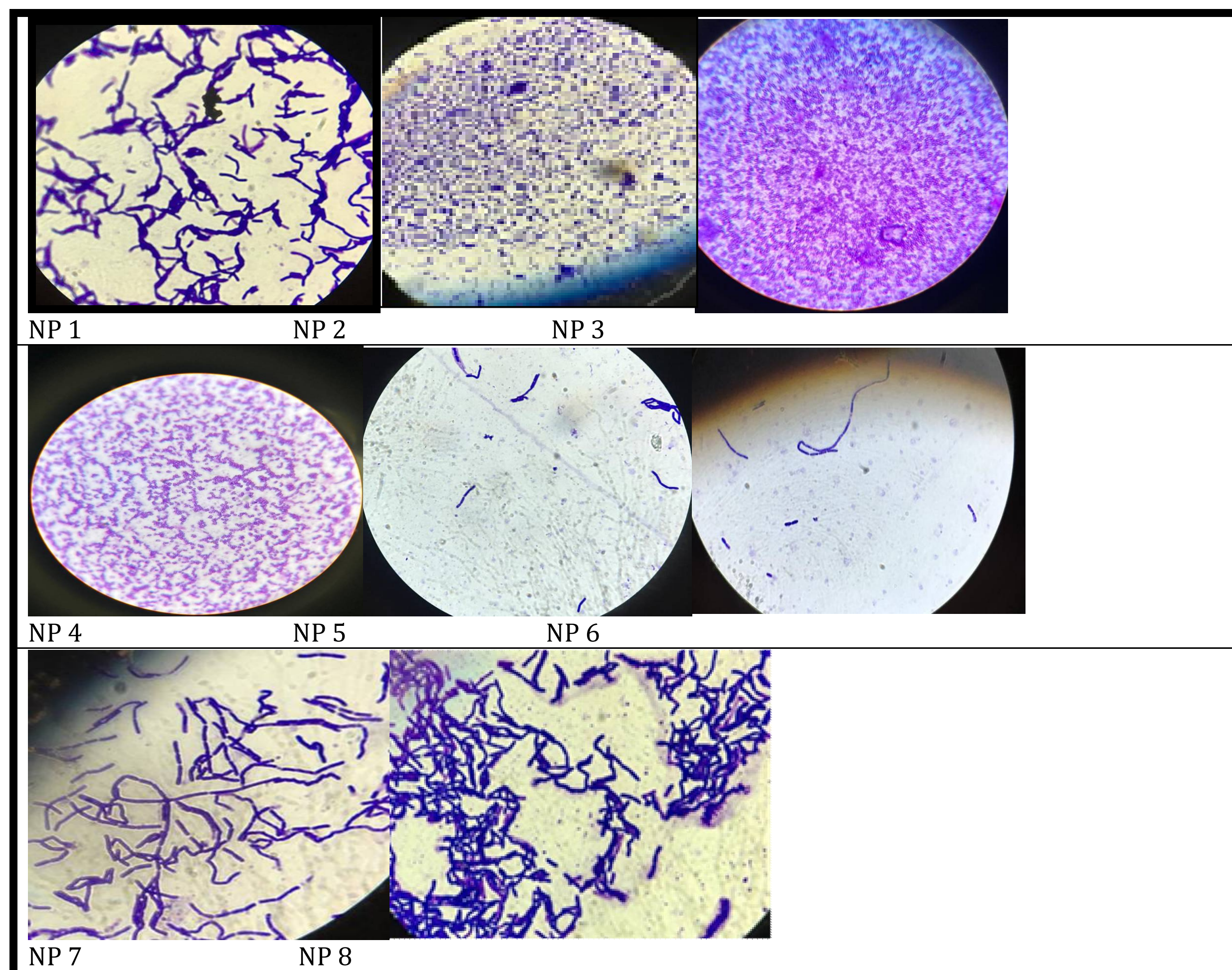

Figure: 3 Microscopic observations of Bacterial strains for pullulanase production

Biochemical characteristics	NP1	NP2	NP3	NP4	NP5	NP6	NP7	NP8
Malonate	+Ve	+Ve	-Ve	-Ve	+Ve	+Ve	-Ve	-Ve
Voges Proskauer's	-Ve	+Ve	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve
Citrate	+Ve	+Ve	+Ve	+Ve	-Ve	+Ve	+Ve	+Ve
ONPG	+Ve	+Ve	-Ve	+Ve	-Ve	+Ve	-Ve	+Ve
Nitrate reduction	-Ve	+Ve	-Ve	+Ve	-Ve	-Ve	-Ve	-Ve
Catalase	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve
Arginine	+Ve	-Ve	-Ve	-Ve	-Ve	+Ve	+Ve	+Ve
Sucrose	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve
Mannitol	+Ve	-Ve	+Ve	-Ve	+Ve	+Ve	+Ve	-Ve
Glucose	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve
Arabinose	-Ve	-Ve	+Ve	-Ve	+Ve	-Ve	-Ve	-Ve
Trehalose	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve

Table 3: Summary of Biochemical characteristics for isolated bacterial strains

Here, '+' Sign indicate Positive result; '-'sign indicate Negative result

By examining morphological, microscopic, and biochemical test results, the organisms identified as *Bacillus* species.

Molecular identification of isolates:

For a number of years, the 16S rRNA gene has served as a cornerstone of sequence-based bacterial study. Taxonomic resolution at the species and strain levels is provided by the 16S gene [14].

The bacterial culture with the highest pullulanase activity was chosen for 16S rRNA sequencing as 16S is genetic marker present in all bacteria, which can help in the identification of bacterial species and also in construction of phylogenetic tree to identify the position of the selected bacterial culture [6, 12].
In this study, 27 F/1492 R primer was employed. Obtain FASTA sequences of Query length is 1555 bases submitted to NCBI (Figure 4).

Bacillus subtilis strain NP 7 16S ribosomal RNA gene, partial sequence

GenBank: OP615140.1

FASTA Graphics

Go to:

```
LOCUS       OP615140               1555 bp    DNA     linear   BCT 17-OCT-2022
DEFINITION  Bacillus subtilis strain NP 7 16S ribosomal RNA gene, partial
            sequence.
ACCESSION   OP615140
VERSION     OP615140.1
KEYWORDS    .
SOURCE      Bacillus subtilis
  ORGANISM  Bacillus subtilis
            Bacteria; Bacillota; Bacilli; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 1555)
  AUTHORS   Patel,N. and Padhiar,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (12-OCT-2022) Shri M. M. Patel Institute of sciences and
            research, Kadi sarva vishwavidyalaya, sector 23, gandhinagar,
            gujarat 382023, India
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            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
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                     /mol_type="genomic DNA"
                     /strain="NP 7"
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                     /db_xref="taxon:1423"
     rRNA            <1..>1555
                     /product="16S ribosomal RNA"
```

Figure 4: NP 7 Bacterial strain NCBI submission result (Accession number OP615140.1)

For sequence similarity analysis, the Query sequence was aligned with extremely similar sequences in the NCBI repository using the BLAST (https://blast.ncbi.nlm.nih.gov/Blast) programme. The NP 7 bacterial strain displayed 100% sequence similarity to 15 different deposited *Bacillus substilis* sequences, according to BLAST analysis.
Pullulanase could be produced more easily and safely by *Bacillus subtilis* than by the techniques now in use. Furthermore, because *Bacillus subtilis* is a generally regarded as safe (GRAS) microorganisms-derived product, it is a suitable species for enhancing pullulanase production [18]. *Bacillus subtilis* MF467279 was isolated from the *Salsola imbricate* desert plant species, which exhibits the best pullulanase activity (16.6 U/mL) at 72 h was identified molecularly by Abeer *et al*., (2018) using the 16S rRNA Sequencing method [1].
Mega XI software was used to analysis the phylogenetic position of NP 7 bacterial culture. The bootstrapping technique, invented by Felsenstein which is frequently used in evolutionary biology research was used to build the tree. The sequences of 15 closely related species were aligned to create a rooted tree (Figure 5). The Kimura 2-parameter model and the Maximum Likelihood statistical technique were used to construct the phylogenetic tree [16]. 500 replicates of bootstrapping were utilized to study tree topology. There were 16 nucleotide sequences in this investigation. (Complete deletion option) All places with gaps and incomplete data were removed. The final dataset contained 1554 locations altogether. The produced tree displays the highest log likelihood, which is -2138.97.

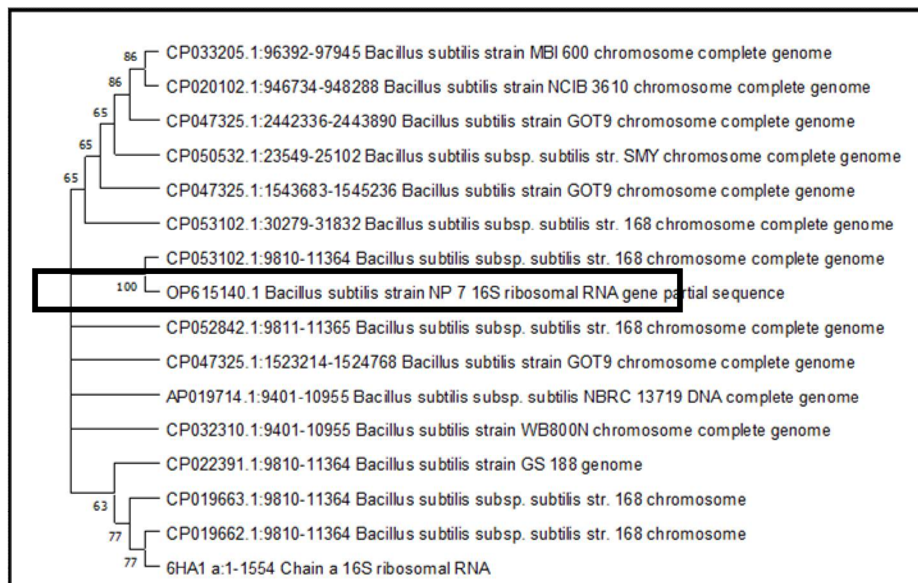

Figure 5: Rooted phylogenetic tree showing the phylogenetic relationship of Pullulanase producing strain (NP 7) with other species

CONCLUSION:

From the research finding, it can be concluded that NP7, isolated from a soil sample produced maximum pullulanase activity of 33.33 U/ml at 24 hours. NP 7 was identified as *Bacillus subtilis* using morphological, biochemical & molecular identification method. Pullulanase production & its activity can be improved by optimizing the culture conditions and media used for cultivation, allowing for further use in research to create industrially significant applications.

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