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BIOPROSPECTING OF BACILLUS PUMILUS NGP-2 FOR THE PRODUCTION OF PROTEINACEOUS α-AMYLASE INHIBITOR

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ABSTRACT

The study was conducted to screen the proteinaceous α-amylase inhibitory activity of *Bacillus pumilus* NGP-2. The culture free supernatant was precipitated by ammonium sulfate at 10-80% saturation for overnight at 4ºC. The precipitated proteins were desalted by dialysis and the protein content was estimated as 0.5mg/ml. The proteinaceous α-amylase inhibitor was subjected to Preparative High Performance Liquid Chromatography. Two fractions were collected and α-amylase inhibitory activity was checked. Fraction I showed α- amylase inhibitory activity of 64%.The molecular weight of the α-amylase inhibitor was determined by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis as 65 kDa. The results suggested that the proteinaceous α-amylase inhibitor from *Bacillus pumilus* NGP-2 may be a potent inhibitor and can find role in the management of post prandial hyperglycemia.

KEYWORDS: ***Post prandial hyperglycemia, α-amylase inhibitor, Diabetes mellitus, Starch blockers, Porcine pancreatic α-amylase.***

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INTRODUCTION

Diabetes is a chronic disease that occurs either when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces. It is characterised by elevated level of blood glucose or chronic hyperglycemia with disturbances in carbohydrate, fat and protein metabolism. Reports reveal 592 million likely to suffer from diabetes all over the world by 2035.[1] India currently has the prevalent figure of diabetic patients worldwide and designated as the diabetic capital of the world.[2] There are two major forms of diabetes type I and type II diabetes. The majority of diabetes is type II diabetes caused by a combination of impaired insulin secretion from pancreatic beta cells and insulin resistance of the peripheral target tissues, especially muscle and liver.[3] Post prandial hyperglycemia is an early defect in type 2 diabetic patients that leads to severe diabetic macro and micro-vascular complications.[4] Delayed insulin secretion immediately after meal results in persistently elevated post prandial glucose (PPG).[5,6] The recent approach for controlling post prandial hyperglycemia is to inhibit the carbohydrate-hydrolysing enzymes such as α-amylase and α-glucosidase in the digestive system.[7] Inhibitors of these enzyme delay carbohydrate digestion and prolong overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently blunting the postprandial plasma glucose rise.[8]Amylase inhibitors are also known as starch blockers because they contain substances that prevent dietary starches from being absorbed by the body. Starch is a complex carbohydrate that cannot be absorbed unless they are first broken down by the digestive enzyme amylase and other secondary enzymes. α-amylase inhibitors are naturally distributed in microorganisms, higher plants, or animals secretions, which play a vital role in the control of α- amylase activity.[9] α-amylase inhibitors are classified as proteinacoeus and non-proteinaceous. The proteinacoeus amylase inhibitors usually exhibit much stronger inhibitory effect than that of non-proteinaceous inhibitors. Therefore it should be a potential candidate for therapeutic application due to their strong amylase inhibition.[8] Inhibitors currently in clinical use are acarbose and miglitol which inhibit glycosidase enzymes. However, many of these synthetic hypoglycaemic agents have their limitations. They are non-specific, produce serious side effects and fail to reduce the diabetic complications.[10] A lot of research is being undertaken in α-amylase inhibitors from plants but the study of α-amylase inhibitor from microbial origin is limited. Microbial source of a bioactive compound is easier and economically viable to produce. Usually microorganisms represent a vast resource of novel compounds with many agricultural and medical applications. This study is focussed on proteinaceous α-amylase inhibitor from *Bacillus pumilus* NGP-2 which would be cost effective and a potential candidate for application in medical area for their strong α-amylase inhibition abilities by eliminating a lot of side effects associated with synthetic drugs.

MATERIALS AND METHODS

Collection of Soil Samples

Soil samples were collected from Valparai, Tamilnadu, India in a sterilized poly bag sealed properly and brought to the laboratory for further analysis. It was stored at 4°C until used.

Isolation of bacteria

One gram of soil was suspended in 100ml sterile distilled water and serially diluted up to10^{-7}. From each dilution 0.1ml of sample was spread plated on nutrient agar plates and incubated at 37°C for 24 hours. Individual colonies were selected based on the colony morphology, appearance and maintained in agar slants.

Screening for α-amylase inhibitory activity

All the isolated bacterial isolates were screened for α-amylase inhibitory activity.[11]500µl of cell free supernatant and 500µl of porcine pancreatic α-amylase solution (0.5 mg/ml) were incubated for 10 minutes at 25ºC. After pre-incubation, 500µl of 1% starch solution was added to each tube. The reaction mixtures were then incubated at 25ºC for 10 minutes. The reaction was stopped with 1ml of dinitrosalicylic acid reagent and incubated in boiling water bath for 5 minutes. The content was cooled to room temperature. The reaction mixture was then diluted after adding 10ml distilled water and absorbance was measured at 540 nm. Control was maintained without the inhibitor. All the reactions were carried out in triplicate value. Percentage of inhibition was calculated using following equation: % Inhibition = [(A_{540} Control – A_{540} Inhibitor) / A_{540} Control] X 100. The isolate which showed the highest percentage of inhibition was taken for further study. The protein content of cell free supernatant was estimated.[12]

Production of α-amylase inhibitor

The isolate *Bacillus pumilus* NGP-2 was inoculated in 100ml BPM6 production medium (0.5g peptone, 0.15g yeast extract, 0.05g magnesium sulfate, 0.5g sodium chloride, 0.15g beef extract, 0.03g dipotassium hydrogen phosphate, pH 7.4±0.2) and incubated at 37°C for 48 hours. It was then centrifuged at 7,000 rpm for 10 minutes and the cell free supernatant was precipitated by ammonium sulfate at 10-80% saturation and kept for overnight at 4ºC. The precipitated proteins were centrifuged at 12,000 rpm for 30 minutes and the pellet was suspended in 0.02M sodium phosphate buffer (pH 6.9). The protein content was estimated for the ammonium sulfate precipitates before desalting.[12] Desalting of protein was carried out by dialysis (MWCO 12000 to 14000) against phosphate buffer saline (pH 7.4) with the buffer change at regular interval of an hour. The α-amylase inhibitory activity and protein content of precipitated proteins with 10-80% saturation after desalting were examined and the saturation percentage which exhibited maximum activity was selected for further study.

Molecular Identification

The bacterial isolate which exhibited the remarkable α-amylase inhibitory activity was identified by 16S rDNA Sequencing.[13]The 16S rRNA gene was amplified using primers 27F (5'AGAGTTTGGATCMTGGCTCAG3'),

1492R (5' CGGTTACCTTGTTACGACTT 3') and was subjected for sequencing.[14] The obtained nucleotide sequence (356bp) was analyzed and submitted to Genbank (Accession number: KY110406).

Partial characterization of proteinaceous α-amylase inhibitor

Analytical and preparative HPLC

The proteinaceous α-amylase inhibitor was subjected to analytical and preparative HPLC (Shimadzu C-18) using UV detector at 220nm with the flow rate of 1ml /minute for the partial purification. Acetonitrile was used as the solvent. After preparative HPLC two fractions were collected and evaporated. Fractions were estimated for protein and screened for α-amylase inhibitory activity.

SDS-PAGE analysis

SDS-PAGE analysis was carried out with 10% separating gel and 5% stacking gel.[15] The protein sample mixed with loading dye was loaded into each lane along with the protein marker of 10-245 kDa in the adjacent well. The gel was stained for proteins with Coomassie Brilliant Blue R-250 and the destained gel was visualized for the bands.

Enzyme Kinetics of α-Amylase inhibitor from Bacillus pumilus NGP-2

The mode of inhibition of the inhibitor against α-amylase was done by using starch as substrate at increasing concentrations (1%,2%,3%,4%,5%) in the absence or presence of the inhibitor. Mode of inhibition was determined by Michaelis-Menten kinetics and Lineweaver- Burk plot analysis.[16]

RESULTS

37 bacterial isolates with different colony morphology were isolated from soil samples collected from Valparai, Tamilnadu, India. All the bacterial isolates were screened for α-amylase inhibitory activity and the cell free supernatant of the isolate *Bacillus pumilus* NGP-2 showed remarkable inhibitory effect of 58% against α-amylase with starch as substrate. The protein concentration of cell free supernatant was estimated as 1.4mg/ml. The isolate was confirmed as *Bacillus pumilus* NGP-2 (KY110406) by 16S rDNA sequencing (Figure1). 40% saturated ammonium sulfate precipitates showed highest inhibitory activity of 62.27% with protein concentration of 0.5mg/ml. In analytical HPLC, two peaks were observed in the chromatogram with retention time of 2.746 and 3.895 (Figure 2). Using preparative HPLC column, two peaks were fractionated. Fraction I inhibited porcine pancreatic α-amylase enzyme (64%) than the fraction II. The protein content of fraction I was estimated as 0.4mg/ml. Fraction I from the preparative column was found to contain one major band with a molecular weight of 65 kDa when analyzed by SDS-PAGE (Figure 3). The Lineweaver Burk plot revealed the inhibition pattern of partially purified α-amylase inhibitor as uncompetitive mode of inhibition with the K_m value of 5.79 for without inhibitor (0 mg), 5.67 for inhibitor with 0.25mg/ml, 5.31 for inhibitor with 0.5mg/ml and V_{max} of 0.996 for without inhibitor (0 mg), 0.582 for inhibitor with 0.25 mg/ml, 0.467 for inhibitor with 0.5 mg/ml using starch as substrate (Figure 4 and 5).

GGCAGGTGGCGGCTTGCCAATACTGGCAAGTCGAGCGGACAGAAGGGAGCTTGCTCCCGGATGTTAGCGGCGG
ACCGGAGAATAGGGCGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGAGCTAATACCGGATA
GTTCCTTGAACCGCATGGTTCAAGGATGAAAGACGGTTTCGGCTGTCACTTACAGATGGACCCGCGGCGCATTA
GCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCCCAATGGACGAAAGTCT

Figure 1
16S rDNA Sequence of Bacillus pumilus NGP-2

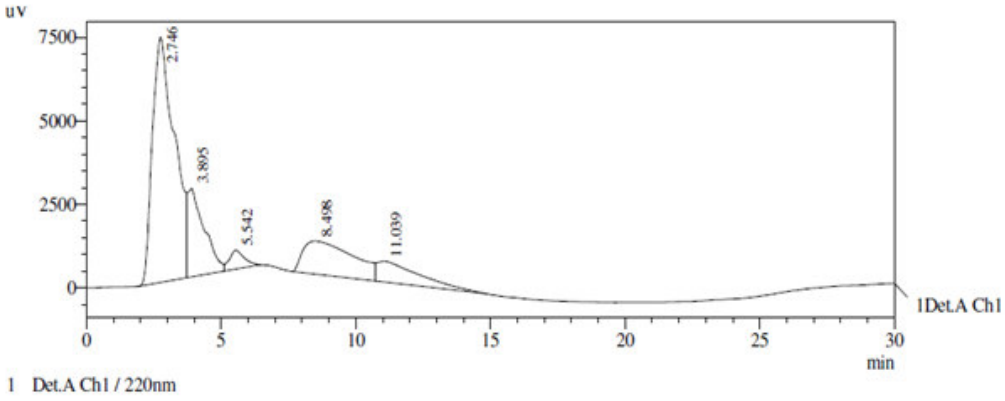
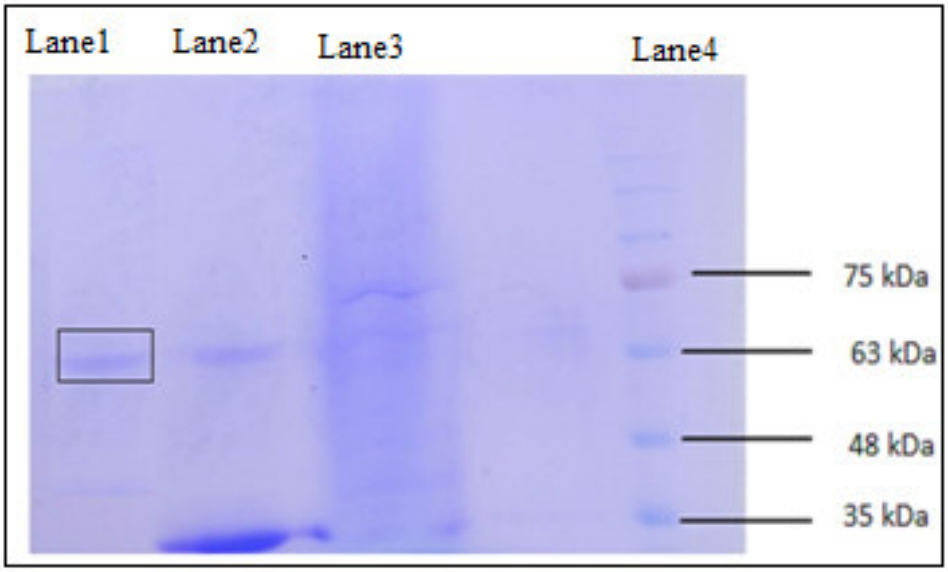


Figure 2
HPLC Chromatogram of α-Amylase inhibitor from Bacillus pumilus NGP-2



Lane 1- Fraction 1 from preparative HPLC
Lane 2- Dialyzed α-amylase inhibitor
Lane 3- Crude protein from *Bacillus pumilus* NGP-2
Lane 4- Protein Marker

Figure 3
SDS PAGE Analysis of of α-Amylase inhibitor from Bacillus pumilus NGP-2

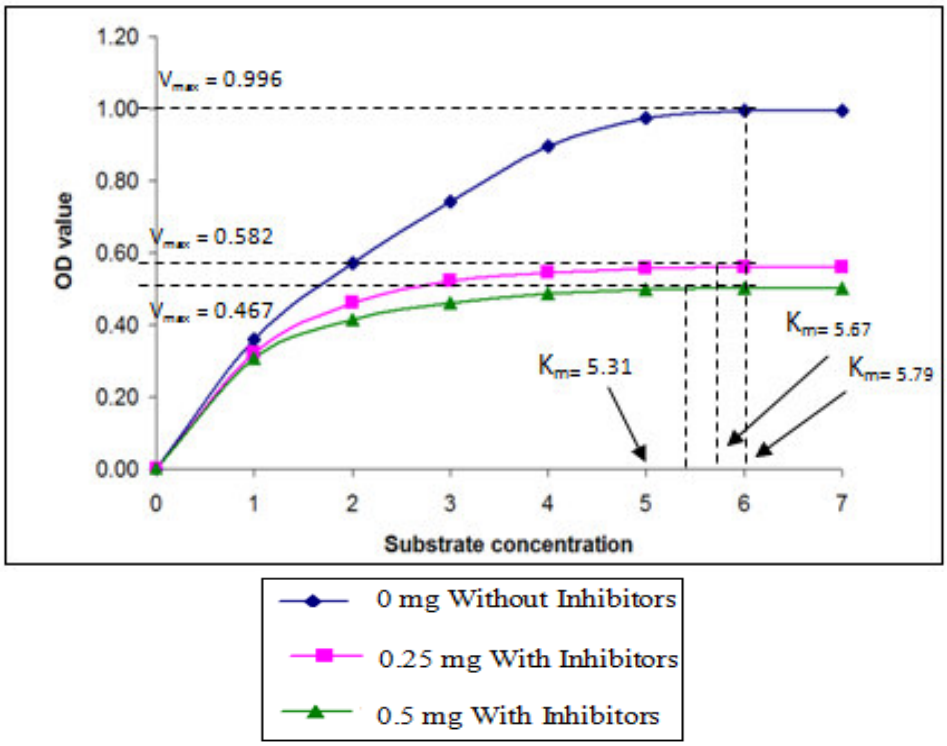


Figure 4
Michaelis Menten Plot of α-amylase inhibitor from Bacillus pumilus NGP-2

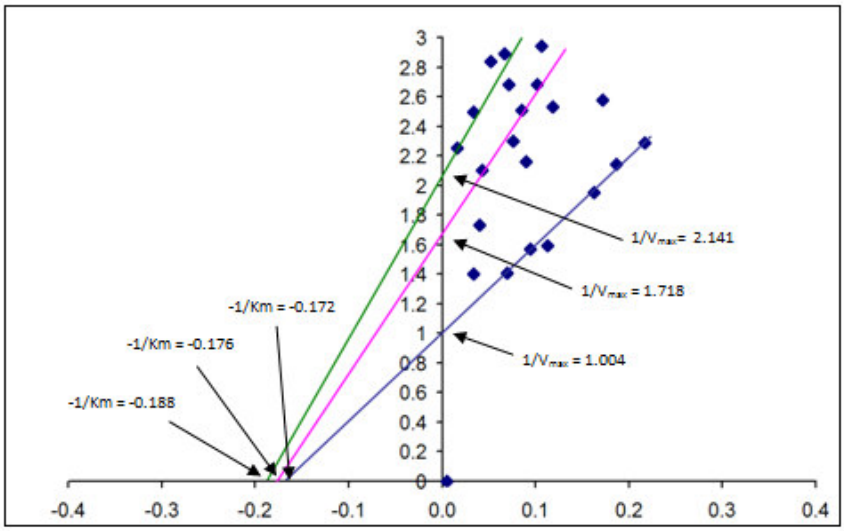


Figure 5
Lineweaver - Burk Plot of α-amylase inhibitor from Bacillus pumilus NGP-2

DISCUSSION

Diabetes is fast gaining the status of a potential epidemic in India. Lots of human populations are affected with type II diabetes. It is caused due to insufficient production of insulin by pancreas or body cells become resistant towards insulin.[17] The early defect in type II diabetic patient is post prandial hyperglycemia. The common strategy for treatment is focused mainly on regulating and decreasing blood sugar to fall within the normal level. The recent approach in controlling post prandial hyperglycemia is to inhibit the enzyme such as alpha amylase.[7] Inhibitors like acarbose, voglibose and miglitol has been used commercially as drug. But these drug have their own limitations due to the side effects like gastrointestinal disturbances. For this reason, it is necessary to produce inhibitors with no or less side effects. Therefore search for new alpha amylase inhibitors from natural resources has become an attractive approach for the treatment of postprandial hyperglycemia. Many plants have been reported for their antidiabetic activities but the alpha amylase inhibitor form microorganisms have not gained much importance in this modern era.In the present study we have isolated *Bacillus pumilus* NGP-2 from Valparai, Tamilnadu, India. By 16s rDNA sequencing the isolate was confirmed as *Bacillus pumilus* NGP-2. In this study, alpha amylase inhibitory activity of *Bacillus pumilus* NGP-2 is extensively studied in an attempt to screen for proteinaceous inhibitors of alpha amylase. Recently *Bacillus pumilus* NGP-1 was reported for its efficient production of proteinaceous alpha amylase inhibitors.[18] The precipitated protein showed a significant alpha amylase inhibitory activity with 62.27%. Similar alpha amylase inhibitory activity was reported on *Streptomyces variabilis* strain PO-178.[19] Previous studies reported alpha amylase inhibitors from *Cladosporium herbarum* F- 828 and *Paenibacillus lentimorbus*.[20,21] Earlier the proteinaceous alpha amylase inhibitor has been reported from common bean seeds.[22]Previous studies were reported on alpha amylase inhibitor producing actinomycetes. Similar observation were reported on *Streptomyces diastaticus* subsp *amylostaticus* No.2476.[23] This study supports the earlier report on proteinaceous alpha amylase inhibitor AA1-CC5 from *Streptomyces* sp CC5.[24] The antidiabetic potential of a peptide from *Aspergillus awamori* was reported.[25] The characterization of partially purified proteinaceous alpha amylase inhibitor was carried out with SDS PAGE analysis. Molecular weight of the proteinaceous alpha amylase inhibitor was determined as 65 kDa. Similarly alpha amylase inhibitory peptide of 22 kDa from *Aspergillus awamori* has been reported.[25] Recently proteinaceous alpha amylase inhibitor of 29 kDa is reported from *Bacillus pumilus* NGP1.[18]Alpha amylase inhibitor of 18 kDa from *Cladosporium herbarum* has reported.[21] Mode of inhibition of partially purified proteinaceous alpha amylase inhibitor was determined by means of Lineweaver burk plot analysis of data according to Michaelis Menten kinetics. The mode of inhibition of proteinaceous alpha amylase inhibitor appeared to be uncompetitive (Km and Vmax value decreases). Similarly uncompetitive mode of inhibiton was reported.[16] Earlier study reported the uncompetitive inhibition pattern for alpha amylase inhibitor from *Dioscorea esculenta*.[26]

CONCLUSION

The present study concluded that proteinaceous alpha amylase inhibitor from *Bacillus pumilus* NGP-2 will be greatly beneficial to reduce the rate of absorption of carbohydrate and there by contribute to effective management of diabetes by decreasing post prandial hyperglycemia. Future studies will provide an insight for the biomolecule characterization and molecular mechanism of alpha amylase inhibitor.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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