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Research Article

Screening and Optimization of α-Glucosidase Inhibitor Production by Potent Strain of *Bacillus subtilis* Isolated from Peruyaan, Fermented Soy-Food of Northeast India

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Background. Fermented soy foods exhibit the capability to inhibit the α-glucosidase enzyme. The bacteria isolated from these fermented soy foods may contribute to the production of a higher quantity of α-glucosidase inhibitor (α-GI) in optimized condition. *Aim*. The present study aims to isolate α-glucosidase inhibitor producing bacterial strain from peruyaan, a traditional fermented soy food of Arunachal Pradesh, optimize the production of α-GI for maximum yield, and assess the compounds. *Result*. *Bacillus subtilis* PM NEIST_4 (identified by 16S rRNA gene sequencing) was isolated as a potential strain that exhibit maximum α-glucosidase inhibition. This strain showed maximum α-GI productivity in Mueller Hinton Broth (MHB). The cultivation parameters for fermentation of MHB were optimized in order to maximize the yield of α-GIs. The α-GI productivity of 1616.613 ± 84.54 U/mL was observed before optimization, which increased by almost three times after optimization (4808.324 ± 13 U/mL). *In vitro* assays against α-glucosidase enzyme revealed a significant IC_{50} value of 596.532 ± 44.80 μg/mL after the optimized study compared to that before optimization (IC_{50} 1705.617 ± 43.95 ug/mL). Besides, α-glucosidase enzyme inhibitory property of fermented MHB (FMHB) was found to be unaffected at varied range of pH and temperature. Oral gavaging of FMHB in sucrose-loaded Wistar rat exhibited maximum reduction in postprandial blood glucose at 400 mg/kg body weight. Furthermore, FMHB was fractionated using solvents of increasing polarity including n-hexane, ethyl acetate, and n-butanol. Ethyl acetate fraction that exhibited the strongest inhibitory action against the α-glucosidase enzyme (IC_{50} value of 251.55 ± 19.65 μg/mL), showed the presence of pyrrolopyrazine derivatives through GC-MS and LC-MS analyses. *Conclusion*. The present study demonstrated the isolation of a potent α-GI producing bacterial strain from traditional fermented soy food, and the optimization of the bacterial fermentation process was achieved to maximize the yield of α-GI. These findings underscore promising industrial potential of *B. subtilis* PM NEIST_4, advocating the use of indigenous microbial resources for the production of α-GI.

1. Introduction

Diabetes is a serious metabolic disorder that is highly ranked as a global pandemic on the international health agenda and is characterized by hyperglycemia [1, 2]. Diabetes can be most commonly classified into Type 1 (insulin-dependent diabetes mellitus), Type 2 (noninsulin-dependent diabetes mellitus), and gestational diabetes. Type 2 diabetes mellitus is primarily responsible for the current global epidemic of the disease, accounting for more than 90% of the diagnosed cases of diabetes mellitus [3]. However, studies indicate relatively lower epidemiological prevalence of type 2 diabetes within the Asian population, where a higher consumption of fermented soy products is observed [4]. Over

a long period of time, soybean (*Glycine max*) has evolved as a dietary staple in a majority of Asian countries [5]. It is indeed a nutritious source of high-quality protein for human consumption [6]. However, soybeans typically contain certain anti-nutritional factors in addition to some toxic compounds like agglutinin, soyatoxin, proteinase inhibitors, and urease [7]. Interestingly, the fermentation of soybeans facilitates the degradation of some of these antinutrients while also producing small-sized proteins, bioactive peptides, and active isoflavones that exert a favourable effect on a variety of physiological conditions, such as diabetes [8, 9]. It has been scientifically proven that fermented soybeans help to combat cancer and tumors as well as protect against cardiovascular diseases, neurodegenerative diseases, inflammation, obesity, and diabetes [10]. Most of the Asian countries, including India, have a wide practice of fermenting foods with *Bacillus* species [11]. The type of food might differ depending on the country; however, the use of *Bacillus* for fermentation is quite common. Fermentation of soybeans with *Bacillus* produces viscous substances to finally yield Natto (Japan), Chongkukjang (Korea), Pepok (Myanmar), Sieng (Cambodia, Laos), and Thua nao (Thailand) [12]. In North-Eastern region of India, consumption of fermented soybeans is an age-old tradition followed by various ethnic groups since the origins in Mongolia [8]. Aakhone (Nagaland), Bekang (Mizoram), Hawaijar (Manipur), Kinema (Sikkim), Tungrymbai (Meghalaya), and Peruyaan (Arunachal Pradesh) are some of the naturally fermented soybean foods prevalent in different parts of North-East (NE) India. It is noteworthy that in all these fermented soybean foods; *B. subtilis* is the most predominant [13]. *B. subtilis* has been the subject of extensive research for a very long time, and hence, it is currently one of the well characterized low-GC Gram positive bacteria [14]. As a multifunctional probiotic organism, *B. subtilis* has been an ideal choice for numerous applications ranging from nanoparticle biosynthesis [15, 16] to the production of enzymes [17–20], vitamins [21, 22], antibiofilm, and antimicrobial materials [23]. At the same time, a few studies on probiotic *B. subtilis* unraveled a new dimension revealing its antimicrobial, antiviral, and anticancer properties [24]. Food and Drug Administration (FDA) has acknowledged *B. subtilis* as a GRAS (Generally Recognized as Safe) organism [25]. In addition, *B. subtilis* is widely known for its superior fermentation properties, substantial product yields (20–25 g/L), and the complete absence of harmful by-products [26]. However, the microbial system produces a very low amount of products in fermentation which poses a challenge to separate them for industrial preparations. Consequently, in order to achieve the highest possible yield of the desired product, the fermentation conditions must be improved [27]. To find the ideal relationship between the fermentation rate and the independent variables, statistical optimization is a helpful tool for analyzing the interactions between various elements. It is a set of statistical and mathematical methods that have been applied to the development, enhancement, and optimization of different processes in order to maximize biomass output or bacterial harvests [28]. Recent studies have revealed that bacterial strains, such as *Bacillus amyloliquefaciens* SY07 [29], *Bacillus amyloliquefaciens* AS385 [30], *Bacillus methylotrophicus* K26 [31], *Bacillus subtilis* B2 [32], and *Paenibacillus* sp. TKU042 [33] have the capability of producing α-Glucosidase inhibitors (α-GIs) directly into the fermentation broth. Inhibition of α-glucosidase activity is considered to be a well-established strategy for managing postprandial hyperglycemic pathophysiology linked with type 2 diabetes, delaying the digestion and absorption of oligosaccharides by competitively blocking α-glucosidase enzyme at the brush border of small intestine [34, 35]. Several commercially available α-GIs, like acarbose, voglibose, and miglitol, have been clinically used to treat diabetes mellitus because of their ability to efficiently control postprandial blood glucose level [36]. However, these treatments accompany some undesirable side effects, such as diarrhea, nausea, vomiting, and may cause potential harm to the kidneys and liver due to which safer and natural α-GIs need to be explored and studied further [37]. The present study aims to isolate α-GI producing bacterial strain from peruyaan, a traditional fermented soy food, optimize the production of α-GI for maximum yield and assess the compounds via GC-MS and LC-MS analyses.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals/Reagents and Solutions. Nutrient Broth (NB), Nutrient Agar (NA), Brain Heart Infusion Broth (BHI), Mueller Hinton Broth (MHB), Tryptone Soya Broth (TSB), Luria Bertani Broth (LB), Sodium chloride, Sodium carbonate, Sodium bicarbonate were purchased from Himedia, India. Gram staining kit (77730-1KT-F), 10X Phosphate-Buffered Saline (PBS), Saccharomyces cerevisiae α-glucosidase (G5006), 4-Nitrophenyl α-D-glucopyranoside (N1377), acarbose (A8980), RPMI media (R1383), and FBS were purchased from Sigma, USA. Streptomycin and penicillin were purchased from GIBCO. Analytical grade solvents including n-hexane, ethyl acetate, and n-butanol were purchased from Fisher Chemical. Biochemical analysis kits (Robonik) are sourced from India.

2.1.2. Equipments. Weighing Balance (UniBiocAP, Shimadzu), High-Pressure Steam Autoclave (EQUITRON, Medica instrument), pH Meter (LAQUA, Horiba), Laminar air flow (Optics Technology), Shaking incubator (Kuhner Lab-Therm LT-XC), Compound microscope (Leica MD750, ICC50), Field Emission Scanning Electron Microscope (STEM, JEOL, JEM 2100 Plus), Freeze dryer (FreeZone, Labconco), Microplate Reader ((Epoch 2, BioTek)). Thermal Cycler (Applied Bio Systems, ThermoFisher Scientific), Nucleic Acid Electrophoresis Gel System (Bio-Rad), Molecular Imager (ChemiDoc Imaging System, Bio-Rad, US), Biosafety level −2 laminar air flow cabinet (1300 SEREIES A2, Thermo Scientific), CO_2 incubator (CellXpert, eppendorf), Liquid chromatography-mass spectrometry (Agilent), Gas chromatography-mass spectrometry (Agilent), Biochemical Analyzer (AutoLab VerSA, Euro Diagnostic System).

2.2. Sample Collection. The fermented soybean, peruyaan, was obtained from a local market in Arunachal Pradesh, India. To ensure a completely sterile environment, the sample was delicately enclosed within a pristine zip-lock plastic pouch and then kept at a temperature of −20°C until it underwent analysis for microbiological evaluations.

2.3. Isolation of Bacteria from Peruyaan. A quantity of 10 g of peruyaan from the stock was delicately ground with a previously sterilized mortar and pestle, then transformed into a harmonious mixture by suspending it in 90 mL of sterile 0.9% NaCl solution followed by vigorous agitation for a duration of 60 min, which was serially diluted 10-fold over a range of 10^{-1} to 10^{-6} in sterilized test tubes. A 100 μL from each diluted suspension were individually plated onto distinct nutrient agar plates, each plate meticulously designated for its corresponding dilution level. The plates that were introduced with the microbial specimens underwent an incubation period of 24 h at a temperature of 37°C, facilitating the emergence of well-defined colonies. These colonies, characterized by their unique morphological attributes including shape and texture, were subsequently isolated and transferred onto fresh nutrient agar plates through subculturing, thereby ensuring the cultivation of pure and unadulterated clusters.

2.4. Screening of α-GI Producing Bacteria. Based on α -glucosidase inhibition activity, the potent bacteria was selected. Therefore, for the screening procedure, isolated bacteria were separately incubated (final $OD_{600} = 10^{-3}$), in 250 mL conical flask having 100 mL NB medium (1.3%) as the only source of carbon/nitrogen and allowed to undergo fermentation at a cultivation temperature of 37°C using a shaker incubator at 125 rpm for 72 h [28].

2.5. α-Glucosidase Inhibition Assay. The evaluation of the potential of the experimental samples to inhibit α -glucosidase was conducted using the previous outlined methodology with some modification [38]. In a concise manner, different volumes of supernatant of the fermented culture media sample were used for the assay, making a total of 50 μL with the vehicle. This 50 μL sample mixture was pre-incubated with 25 μL of α-Glucosidase enzyme (at a concentration of 0.5 U/mL) for a duration of 15 min. As a positive control, samples were replaced with 50 μL of acarbose at varying concentrations. Subsequently, 50 μL of the substrate 4-Nitrophenyl α-D-glucopyranoside (at a concentration of 5 mM) in phosphate buffer (pH 6.8) was introduced to initiate the reaction. The amalgamation was then subjected to an incubation period of 30 min at 37°C. In the end, a halt to the reaction was achieved by introducing 50 μL of a solution containing sodium carbonate (Na_2CO_3) at a concentration of 100 mM. The absorbance was subsequently measured at 405 nm using microplate reader. The α-glucosidase inhibition was calculated by using the formula: percent (%) inhibition= $((A - B)/A) \times 100$.

Where A is the optical density (OD) of reaction mixture containing vehicle and B is the OD of reaction mixture containing test sample with vehicle. Furthermore, the inhibition was expressed in terms of unit activity/mL (U/mL) and IC_{50} value. One U was calculated with the volume of the test sample required for 50% inhibition of the enzyme and IC_{50} value was calculated with the concentration of the test sample required for 50% inhibition of the enzyme. The experimental samples were freeze dried and later dissolved in respective vehicle to make different concentration of the samples to determine its IC_{50}. The measurement was carried out in triplicate.

2.6. Molecular Identification of Bacterial Strain. The 16S ribosomal RNA gene sequence analysis was chosen as the method of choice for identifying the potent bacteria. In order to accomplish this, genomic DNA was isolated employing the phenol-chloroform method with certain modification [39]. Agarose gel electrophoresis and spectrophotometric analysis were used to qualitatively and quantitatively validate extracted genomic DNA. Following that, the genomic DNA underwent targeted sequence amplification using primers 27F (GTTTGATCCTGGCTCAC) and 1492R (GGTTAC CTTGTTACGACTT) in an automated thermal cycler (Applied Bio Systems, ThermoFisher Scientific). The amplicons were visualized on a 1% agarose gel having EtBr, and the sequencing was conducted by Eurofins Genomics India Pvt. Ltd. In the end, the sequence acquired was subjected to a comparison using BLAST (basic local alignment search tool) and subsequently submitted to the GenBank sequence database at NCBI to obtain an accession number. Besides, a phylogenetic tree was also constructed using Maximum Likelihood method.

2.7. Optimization of Cultivation Parameters for Production of α-GIs. Five different media, including NB, BHI, MHB, LB, and TSB were used to culture potent bacteria. Considering this, a temperature of 37°C with an agitation speed of 125 rpm and a 100/250 mL medium/flask volume ratio were set as the cultivation parameters for 9 days. Since MHB resulted to be the most favorable for the production of α-GIs, therefore it was selected for optimization of the fermentation parameters, that includes cultivation temperature (25, 30, and 37°C), media volume (50, 100, 150, and 200 mL), and the amount of seed culture ($OD_{600} = 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}$). The culture supernatant of each of the fermented media was allowed to go under centrifugation at 4000 rpm for 20 min, followed by filtration, and then utilized to assess the inhibitory activity of yeast α-glucosidase [33].

2.8. Measurement of Inhibitor Stability. The pH stability of the inhibitor were determined by mixing FMHB with HCl to decrease the pH or mixed with NaOH for increasing it. In particular, 0.5 mL of FMHB (2 mg/mL) were treated with a pH range from 1 to 13 at 37°C for 30 min and subsequently, its α-Glucosidase inhibition efficacy was evaluated at pH 6.8

[40]. To evaluate the thermal stability of the inhibitors, the samples were subjected to a temperature range of 30°C to 120°C for a duration of 30 min. Following this, the inhibitory activity of the sample against yeast α-Glucosidase was assessed [40].

2.9. Cell Culture. HCT-8, human ileocecal adenocarcinoma cells were provided by ATCC, USA and thereafter cultured in RPMI (Sigma) media, supplemented with solutions, including 10% FBS, 1% streptomycin, and 1% penicillin. The cells were maintained under incubation at 37°C and 5% CO_2 [41]. The cells were allowed to grow in a standard T25 flask until confluency reached to 80–90%. The cells were trypsinized prior to confluency and the culture medium was changed at every alternate day. At 1×10^5/mL cell density, the cells were further seeded onto experimental plates.

2.9.1. Cytotoxicity Assay. After 90% confluency was achieved, HCT-8 cells were treated with different concentrations of FMHB for determining its cytotoxic effect. In particular, the cells were pretreated with 0, 1, 2.5, 5, and 10 mg/mL concentration of FMHB (as treatment groups) and PBS (as a control) for 20 h. After the treatment, cell culture media was discarded and Alamar blue reduction bioassay was performed to evaluate cell cytotoxicity [42].

2.10. Animal Study. Adult male Wistar rats were obtained from the Department of Veterinary Parasitology, College of Veterinary Science, Guwahati, Assam. The animals were given a two-week acclimatization period under laboratory conditions before the commencement of the experiments. The rats were kept in polycarbonate cages, housed in a clean room maintained at a temperature of 22–24°C with a 12 h light/dark cycle and relative humidity at 55 ± 5%. The animals were provided with a standard pellet diet and regular drinking water. The animal study was approved by the Institutional Ethical Committee and CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals), Ministry of Environment and Forests, New Delhi, India [2028/G)ReBi/S/18/CPCSEA].

2.10.1. Acute Toxicity Study. The oral acute toxicity study was conducted following the guidelines of Organization for Economic Co-operation and Development (OECD) [43]. A total of two groups of Wistar rats, five in each, weighing 75 ± 5 g were selected for this study. The FMHB extract was orally administered to each of the five animals with a single dose of 2000 mg/kg body weight (BW), and control group was administered with normal water. The animals were observed individually for the first 5 min to avoid any clinical sign of concern. Later, each of them were checked upon after every 30 min for the first 6 h after dosing, followed by periodic observation for 24 h and, daily thereafter, for a total of 2 weeks to detect any mortality and behavioral changes that include food intake, body weight, drinking water, urination, faeces, abnormal respiration, salivation, lacrimation, skin texture and color, eye color, abnormal jumping, head twitches, scratching, Straub tail, loss of balance, drowsiness, and aggressiveness. The body mass of the animals were also measured on day 1, day 7, and day 14.

2.10.2. Biochemical Analysis for Liver and Kidney Function Tests. After two-weeks of observation, the rats were sacrificed, and blood samples were obtained via a heart puncture procedure. The collected blood was then processed to extract plasma, which was subsequently utilized for liver and kidney function tests. The levels of alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were quantified to assess liver function. Additionally, creatinine levels were measured to evaluate kidney function.

2.10.3. Effect of FMHB on Blood Glucose in Sucrose-Loaded Rat. The effect of FMHB on the reduction of postprandial plasma glucose in sucrose-gavaged rat was performed according to the experimental animal protocol described by Wresdiyati et al., with some modification [44]. Adult male rats were allocated into six groups, with six animals in each group. All rat had similar average plasma glucose level and BW. The groups were divided as follows: normal group receiving only water, control group receiving normal water and sucrose, a drug control group receiving acarbose and sucrose, and three treatment groups receiving different doses of FMHB (100 mg/kg BW, 200 mg/kg BW, and 400 mg/kg BW) and sucrose. Prior to the experiment, all animal groups underwent an overnight fasting period of 16 h. Subsequently, they were orally administered water, acarbose, and the extract according to their respective experimental groups. After 20 min, a sucrose solution (3 g/kg BW) was orally administered to control, drug control, and treatment groups. Blood glucose level was measured at intervals of 0, 30, 60, 90, and 120 min by glucometer. The changes in blood glucose from the basal level after treatment were analyzed and represented as delta blood glucose level [45].

2.11. α-Glucosidase Inhibition Efficacy of the Solvent Fractions from FMHB. About 600 mL of FMHB was fractionated with different solvents by employing the solvent-solvent partitioning method. The partitioning was performed by using solvents of increasing polarity, that include n-hexane, ethyl acetate, and n-butanol with equal volume of these three solvents (thrice) and then concentrated under reduced pressure at 40°C using a rotary evaporator [46]. Thereafter, the activity of these three concentrated fractions along with residual aqueous fraction was assessed through α-glucosidase inhibition assay and their respective IC_{50} values were calculated.

2.12. Assessment of Compounds in Potent Fraction of FMHB. The fraction showing the highest activity against α-glucosidase inhibition was further analyzed by performing GC-MS and LC-MS analyses to assess the potential compounds.

The GC-MS analysis was performed in Agilent 8890 GC system coupled with Agilent 7010 B GC/TQ mass spectrophotometer and HP-5MS ultra inert column (30 m × 250 μm × 0.25 μm) (Agilent). Helium gas was used as

carrier gas with a flow rate of 1.0 ml/min. The injection temperature was 250°C, and 0.3 μL sample was injected in split mode with a split ratio of 1 : 50. The ion-source temperature was 230°C. The column temperature program was as follows: initially hold for 1 min at 50°C and then raised up to 200°C at the rate of 8°C/min and held for 2 min and finally increased up to 260°C at the rate of 15°C/min and hold for 3 min. The total run time was 28.75 min. Agilent Mass Hunter Qualitative Analysis 10.0 software was used for the data analyses and the compound identification was achieved by using NIST Mass Spectral Database (NIST 17): NIST MS Search v.2.3.

The LC-MS analysis was conducted in a UHPLC system connected with mass spectrometer. The chromatographic separation was carried out in a C18 column (Waters, 2.1 × 50 mm, 1.7 μm) operated at 45°C. The flow rate was maintained at 0.3 mL/min, and 0.1% formic acid in water (solvent A), and acetonitrile (solvent B) were used as mobile phase solvents. The total run time was 5 min, and with the elution conditions: 0.5–2 min, 90% A; 2-3 min, 10–90% B, returned to the initial condition (10% B) for 3–5 min. PDA detector was used to acquire the complete spectrum at each time point in the chromatogram. The generation of ions was achieved by using electron spray ionization (ESI) mode and the mass spectra were acquired in positive mode. The data acquisition and processing were performed in MassLynx software.

2.13. Statistical Analysis. Each experiment was conducted in triplicate, at a minimum, and the findings were depicted as the mean ± standard deviation (SD). The data were statistically analyzed with the application of Sigma Stat software (Jandel Scientific, SanRafael, CA), subjecting them to one-way ANOVA and comparing all the groups using Student–Newman–Keuls post hoc method. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. Isolation and Screening of the Promising Strain. During isolation and screening procedure, 23 of the bacterial colonies were isolated from peruyaan. Among the 23 isolates, 4 isolates showed inhibitory activity against α-glucosidase enzyme and were named as P2 MEK, P4 MEK, P6 MEK, and P8 MEK. Analysis of the α-glucosidase inhibitory activity of these 4 isolates revealed that isolate P6 MEK exhibited the strongest inhibitory activity against α-glucosidase (95.42 ± 0.08%) compared to others (Figure 1). This potent strain gave an IC_{50} value of 1705.617 ± 43.95 μg/mL with α-GI productivity of 1616.61 U/mL after 72 h fermentation (Table 1).

3.2. Identification of the Potent Isolate, P6 MEK. The colony displays a distinctive milky-white, round morphology, characterized by a dry surface (Figure 2(a)). Microscopic examination unveiled the bacterial strain to be Gram-positive (Figure 2(b)). Furthermore, scanning electron microscopy (SEM) analysis revealed the presence of rod-shaped bacteria measuring approximately 2-3 μm in length (Figure 2(c)). For

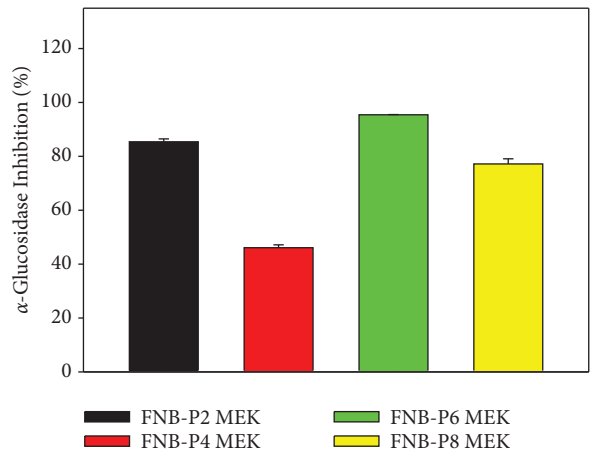

Figure 1: Percentage of α-glucosidase inhibition of nutrient broth fermented individually using four different bacteria isolated from peruyaan, a fermented soybean from Arunachal Pradesh.

molecular identification, the 16S rRNA gene was amplified via PCR and subsequently visualized on a 1% agarose gel (Figure 2(b)). By partial sequencing of 16S rRNA gene amplicons, the isolate was identified as *Bacillus subtilis* PM_NEIST_4 (accession no. OP810678.1) and its consanguinity were analyzed via phylogenetic tree contraction (Figure 2(e)).

3.3. Optimization of Cultivation Parameters for Maximal α-GI Productivity

3.3.1. Effect of the C/N (Carbon/Nitrogen) Source on α-GI Production. To investigate the potency of α-GI production by *B. subtilis* PM_NEIST_4, five sources of C/N, namely 1.3% NB, 3.7% Brain Heart Infusion broth (BHI), 2.1% Mueller Hinton Broth (MHB), 3% Tryptone Soya Broth (TSB), and 2.5% Luria Bertani Broth (LB) were investigated. In order to determine α-GI productivity, the culture supernatants were appropriately diluted to obtain inhibitory activity and then expressed as U/mL. Considering α-GI productivity, significant differences were observed among the five culture supernatants as depicted in Figure 3(a). MHB exhibited the highest α-GI productivity with 1892.34 U/mL on day 6, followed by NB with 1616.61 U/mL on day 3, TSB with 1374.72 U/mL on day 5, LB with 1037.59 U/mL on day 6, and BHI with 624.93 U/mL on day 3, respectively. Considering α-GI productivity, MHB was found as the best source of C/N, and therefore, it was selected for subsequent experiments. Additionally, the cell growth of *B. subtilis* PM_NEIST_4 was also monitored during the process of fermentation by measuring the absorbance of the cell suspension at 600 nm after every 24 h for 9 days. As illustrated in Figure 3(b), no correlation was found between bacterial growth and α-GI productivity.

3.3.2. Effect of Specific Parameters on α-GI Production. In order to achieve maximal α-GI productivity, MHB was selected as the best C/N source to investigate the effect of certain fermentation parameters that include cultivation temperature, culture volume, and concentration of bacteria in terms of ODs.

Table 1: Fermentation conditions and α-GI yield before and after optimization.

Cultivation parameters	Initial conditions	Optimized conditions
C/N source	NB	MHB
Cultivation temperature (°C)	37°C	37°C
Medium/flask volume ratio	2/5	1/5
OD_{600}	10^{-3}	10^{-4}
Cultivation time (day) with maximum α-GI productivity	3rd	6th
α-GI productivity (U/mL)	1616.613 ± 84.54	4808.324 ± 137.26
Inhibition (IC_{50} μg/mL)	1705.617 ± 43.95	596.532 ± 44.80

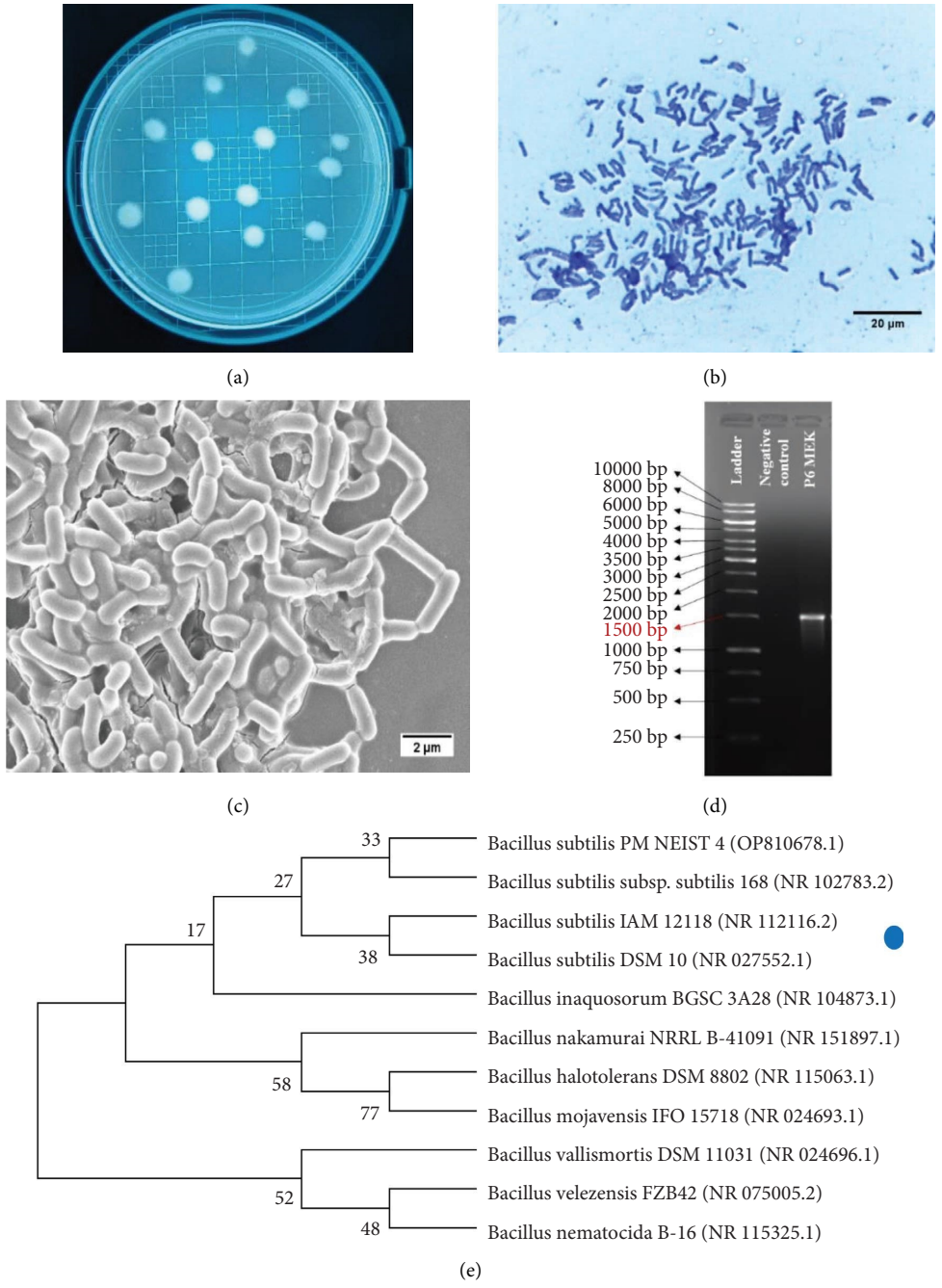

Figure 2: The colony morphology (a); Gram staining image (b); SEM image (c); 16S rRNA gene amplification using 27F and 1492R primers visualized on a 1% agarose gel (d); molecular phylogenetic analysis by maximum likelihood method of potent bacterial isolate, *Bacillus subtilis* PM_NEIST_4 (e).

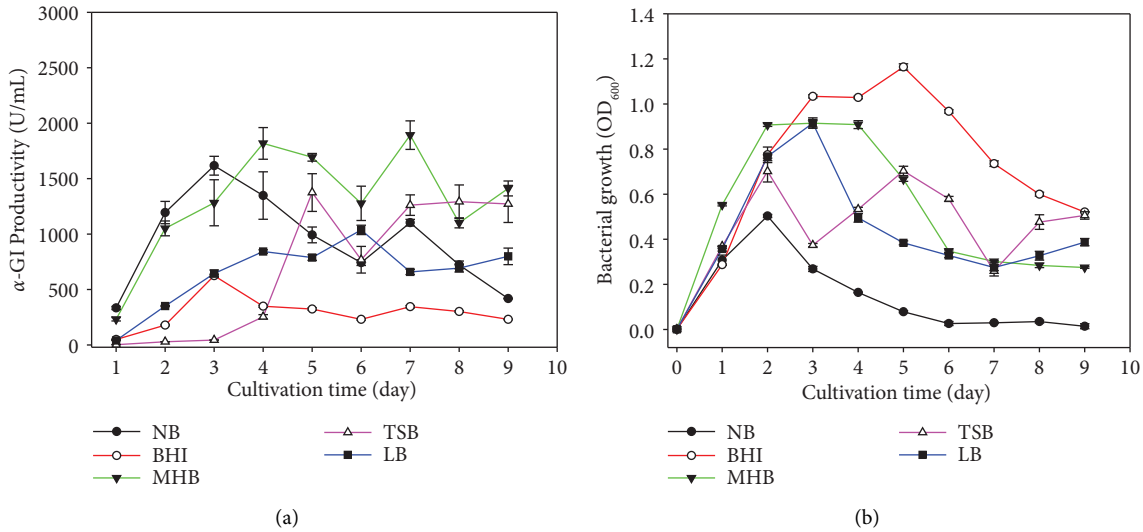

Figure 3: The effect of C/N sources on α-GI production through fermentation with *Bacillus subtilis* PM_NEIST_4 using 1.3% NB, 3.7% BHI, 2.1% MHB, 3.1% TSB, and 2.5% LB as the sole sources of C/N. Fermentation was carried out at 37°C in a shaker incubator set at 125 rpm and medium/flask volume of 100/250 mL at 0.001 OD_{600}. The inhibition against α-glucosidase enzyme was calculated and expressed in U/mL (a); growth of bacterium expressed as OD_{600} (b). Data are expressed as mean ± SD ($n = 3$).

As shown in Figure 4(a), *B. subtilis* PM_NEIST_4 exhibited the highest α-GI productivity (1941.20 U/mL on day 7) at 37°C, approximately 2- and 3.3-fold higher than those at 30°C (884.32 U/mL on day 6) and 25°C (575.18 U/mL on day 2), respectively. Thus, temperature at 37°C was selected as the suitable cultivation temperature to further determine the optimal culture volume for maximum α-GI productivity. Figure 4(b) depicts that α-GI productivity was best achieved in 50 mL medium (4682.46 U/mL) on day 6 of cultivation, followed by 100 mL (1801.63 U/mL), 150 mL (1652.80 U/mL), and 200 mL medium (766.39 U/mL) on 7th day of cultivation, respectively. Consequently, a culture volume of 50 mL medium in 250 mL conical flask (1 : 5 ratio) at a temperature of 37°C was selected to investigate the effect of different OD_{600} of bacteria on α-GI productivity on 6th day of cultivation. The results showed that an OD_{600} of 10^{-4} gave the highest α-GI productivity of 4808.32 U/mL, followed by 10^{-3}, 10^{-2}, 10^{-5}, and 10^{-1} OD_{600} with 3822.88, 3636.49, 3298.148, and 3106.704 U/mL α-GI productivity, respectively (Figure 4(c)). Furthermore, as shown in Figure 5, the IC_{50} value of FMHB after the optimized study was evaluated as 596.532 ± 44.80 μg/mL, which was almost 3 times less than that of FMHB before optimization.

In summary, *B. subtilis* PM_NEIST_4 effectively produced α-GIs in 2.1% MHB-containing media, maintaining an OD_{600} of 10^{-4} at 37°C at a culture volume of 50 mL in 250 mL Erlenmeyer flask, using a shaker incubator at 125 rpm for 6 days. Table 1 represents the fermentation parameters before and after the optimized study.

3.4. Thermal and pH Stabilities of FMHB α-GIs. A temperature range of 30°C to 120°C resulted in FMHB α-GIs being strongly thermostable. The activity was still greater than 95% even when treated at a high temperature of 120°C (Figure 6(a)). At a pH range from 1 to 13, no major differences in α-glucosidase inhibitory activity of FMHB were observed. As shown in Figure 6(b), the stability of α-GI was 91% to 97% at a varied pH range.

3.5. Toxicity Test of FMHB on Cells and Animal Model. To examine the cytotoxicity of FMHB, the Alamar blue bioreduction assay was performed by using intestinal epithelial cells, HCT-8 treated with different concentrations of FMHB (1, 2.5, 5 and 10 mg/mL). The cells treated with PBS (pH 7.4) served as the control. As observed in Figure 7, treatments with FMHB did not show any significant alteration on cell viability even at high concentrations. The findings from acute toxicity studies revealed no unusual behavior, with all subjects demonstrating a 100% survival rate throughout the two-week treatment period (Table 2). Moreover, there were no significant differences in the liver (ALT and ALP) and kidney (creatinine) function tests between normal and those gavaged with FMHB (Table 3).

3.6. Effect of FMHB on Postprandial Blood Glucose Level in Animal Model. To determine the effect of FMHB on postprandial plasma glucose level, three doses of FMHB, 100, 200, and 400 mg/kg BW were administered to male Wistar rats. As illustrated in Figure 8, significant reduction in plasma glucose level were observed for all the three doses administered after sucrose loading. Although, treatment with FMHB at a dose of 100 mg/kg BW demonstrated remarkable effect on suppressing plasma glucose level (Figure 8(a)); however, it was less effective as compared to the higher doses. Treatment with FMHB at a dose of 200 mg/kg BW showed the best reducing effect on blood glucose after 30 and 60 min (Figure 8(b)). At the same time, FMHB at a dose of 400 mg/kg BW reduced blood glucose level maximum after 90 and 120 min effectively

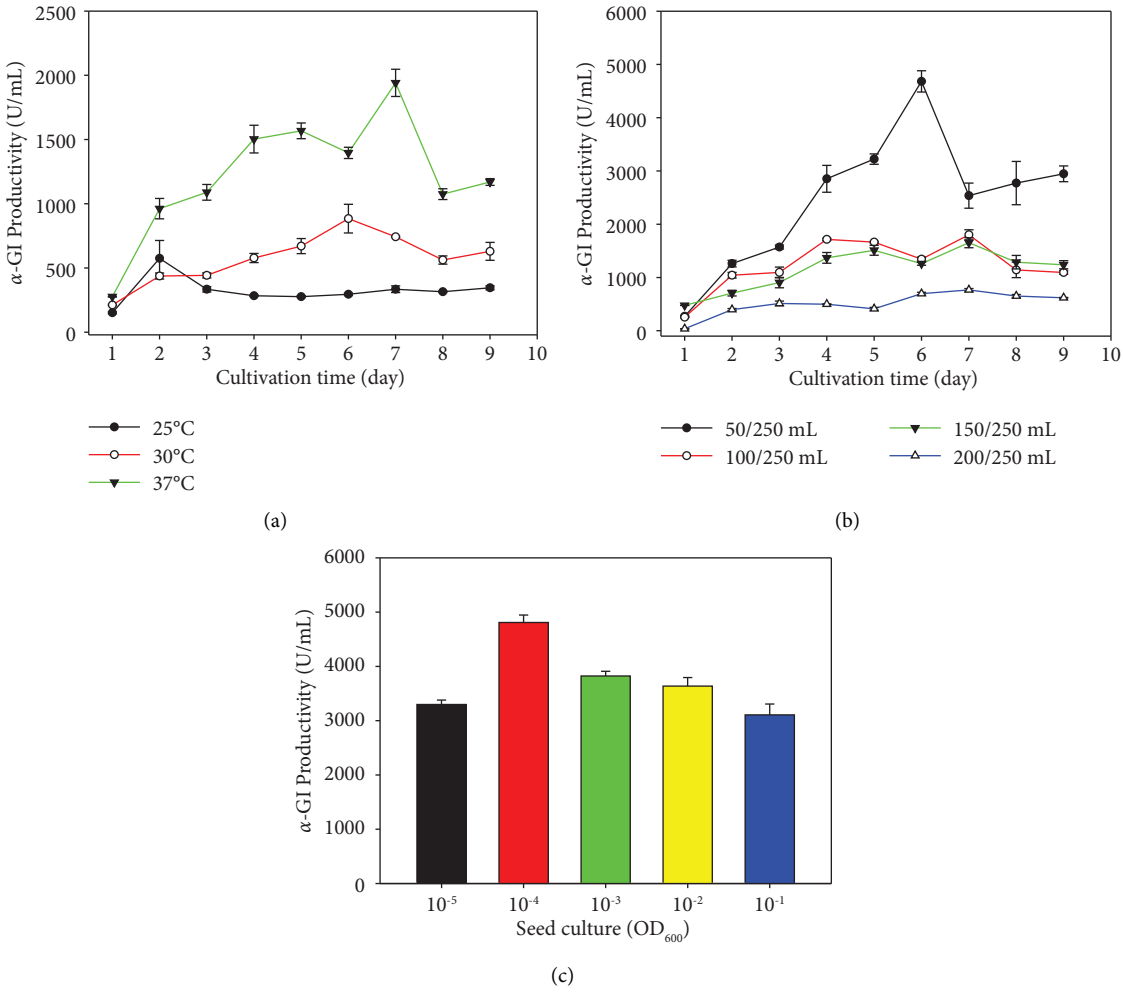

Figure 4: The effect of certain fermentation parameters on α-GI productivity including cultivation temperature (a); medium/flask volume ratio (b); and seed culture (OD_{600}) (c). Data are expressed as mean ± SD ($n = 3$).

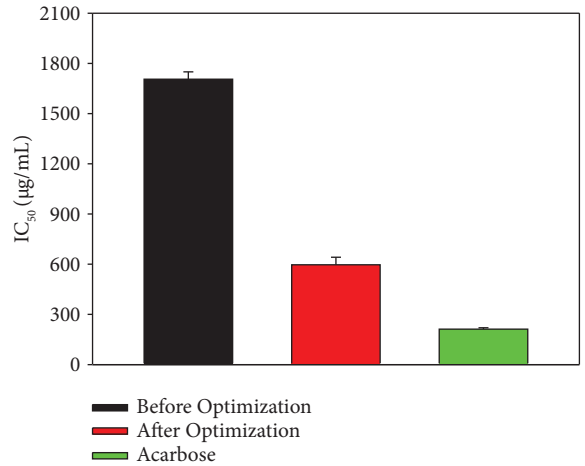

Figure 5: A comparative study between the IC_{50} values of FMHB before and after optimization with the standard, acarbose. Data are expressed as mean ± SD ($n = 3$).

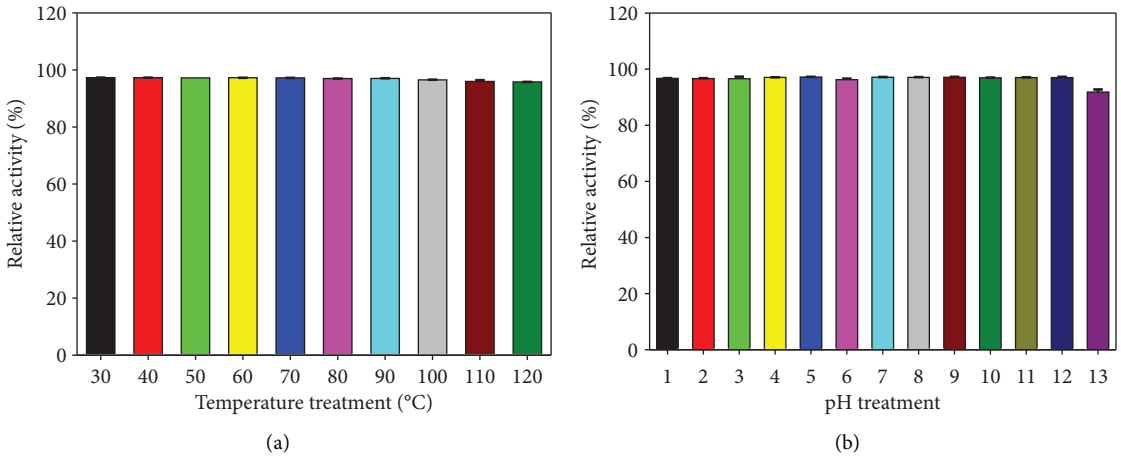

Figure 6: The thermal and pH stabilities of FMHB. Investigating the thermal (a) as well as the pH (b) stabilities of FMHB was done by testing FMHB in the temperature range of 30 to 120°C, and a pH range of 1 to 13 for 30 min, respectively. Data are expressed as mean ± SD ($n = 3$).

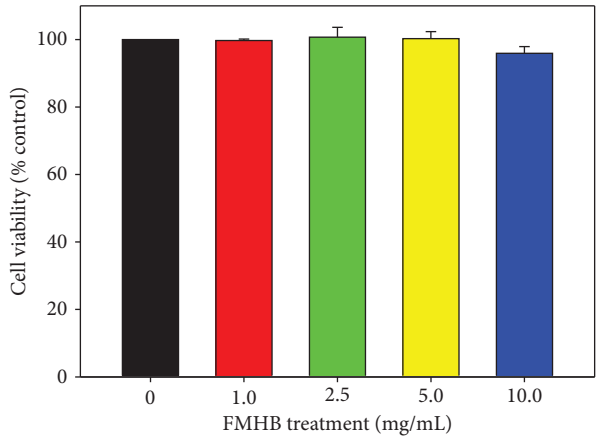

Figure 7: The effect of different concentrations of FHMB (0, 1, 2.5, 5, and 10 mg/mL) on HCT-8, model of intestinal epithelial cells. Data are expressed as mean ± SD ($n = 3$).

Table 2: Behavioral observation of rat in control and FMHB (2000 mg/kg BW) treated group ($n = 5$).

Observed parameters	Control (only normal diet)	FMHB (2000 mg/kg BW)
Food intake	Normal	Normal
Body weight	Normal	No change
Drinking water	Normal	Normal
Urination	Normal	Normal
Faeces	Normal	Normal
Abnormal respiration	Not found	Not found
Salivation	Normal	Normal
Lacrimation	Not found	Not found
Skin texture and color	Normal	No change
Eye color	Normal	No change
Abnormal jumping	Not found	Not found
Head twitches	Not found	Not found
Scratching	Not found	Not found
Straub tail	Not found	Not found
Loss of balance	Not found	Not found
Drowsiness	Not found	Not found
Aggressiveness	Not found	Not found
Death	Not found	Not found

Table 3: The liver (alanine aminotransferase, ALT, and alkaline phosphatase, ALP) and kidney (creatinine) function tests in the control and FMHB (2000 mg/kg BW) treated group.

Parameters	Control	FMHB	Reference level
ALT (U/L)	21.29 ± 2.65	22.47 ± 2.62	10–40
ALP (U/L)	107.17 ± 8.73	105.44 ± 7.68	30–130
Creatinine (mg/dL)	0.506 ± 0.11	0.413 ± 0.071	0.4–0.8

The respective normal range is mentioned alongside [47].

(Figure 8(c)). The test groups showed significant reduction in blood glucose level when compared to the animal treated with sucrose along with standard drug, acarbose at a dose of 50 mg/kg BW (Figure 8(d)). In comparison to animals administered with sucrose alone, the comprehensive glycemic response exhibited a reduction of 34.9%, 67.4%, 69.9%, and 58.9% upon treatment with 100, 200, and 400 mg/kg BW of FMHB, and 50 mg/kg BW of acarbose, respectively (Figure 8(e)).

3.7. Assessment of α-Glucosidase Inhibitory Activity of Solvent Fractions from FMHB. Based on the polarity, four fractions, namely, n-hexane, ethyl acetate, n-butanol, and aqueous fractions were collected from crude FMHB. Among them, the ethyl acetate fraction of FMHB showed the highest α-glucosidase inhibition activity followed by n-butanol, aqueous fraction, and n-hexane. The IC_{50} values of each respective fraction were found to be 251.55 ± 19.65 μg/mL, 985.75 + 51.99 g/mL, 2197.63 + 58.04 g/mL, and 4129.63 + 156.47 g/mL (Figure 9).

3.8. Identification of Compounds in the Solvent Fraction of Bacterial Culture Broth (FMHB) with Promising α-Glucosidase Inhibitory Activity. To identify the compounds responsible for the activity of the ethyl acetate fraction, both GC-MS and LC-MS analyses were performed (Figure 10). The GC-MS analysis of the ethyl acetate fraction demonstrated five major peaks at the acquisition time of 20.629, 22.226, 22.496, 22.677, and 27.145 min (Figure 10(a)). The corresponding GC-MS library revealed the presence of 3-propylhexahydro pyrrolo[1,2-a]pyrazine-1,4-dione (compound 1; $C_{10}H_{16}N_2O_2$), 3-isobutylhexahydropyrrolo [1,2-a]pyrazine-1,4-dione at 22.226, 22.496, and 22.677 min (compound 2, $C_{11}H_{18}N_2O_2$), and 3-benzylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione (compound 3; $C_{14}H_{16}N_2O_2$) (Figure 10(c)). Furthermore, LC-MS analysis of ethyl acetate fraction also revealed the presence of compound 1 (calculated exact mass for $C_{10}H_{16}N_2O_2$ 196.1212, found $[M+H]^+$ 197.1310), compound 2 (calculated exact mass for $C_{11}H_{18}N_2O_2$ 210.1368, found $[M+H]^+$ 211.1494), and compound 3 (calculated exact mass for $C_{14}H_{16}N_2O_2$ 244.1212, found $[M+H]^+$ 245.1331) in the ethyl acetate fraction of FMHB (Figures 10(b) and 10(c)).

4. Discussion

Persistent elevation of blood glucose concentration in diabetic conditions can lead to severe complications such as diabetic nephropathy, neuropathy, retinopathy, hepatic steatosis, cardiovascular disease, and pancreatic disorders [48]. Consumption of fermented soy-based foods has been well known to minimize high blood glucose concentration in diabetic individuals [49]. Daily intake of these fermented food products can help to manage numerous chronic disorders and gain multiple health benefits [49]. In multiple *in vitro*, *in vivo*, and clinical settings, the antidiabetic potential of fermented soybeans has been demonstrated, revealing a wide spectrum of therapeutic targets [49]. Inhibiting key digestive enzymes like α-glucosidase can help to reduce postprandial hyperglycemia associated with diabetes by delaying the digestion of carbohydrates [50]. Studies have revealed that certain glucosidase inhibitors are also found to be naturally present in fermented soybean foods such as tempeh, douchi, doenjang, and miso [51]. However, the knowledge of any such enzyme inhibitor present in fermented soy foods of North-East India is very limited. Previous studies have revealed that a few species of *Streptomyces, Actinoplanes*, and *Flavobacterium* have the capability of producing α-GIs that form the basis of antidiabetic drugs available commercially [29]. In addition, some bacterial strains like *Bacillus amyloliquefaciens* SY07 [29], *Bacillus amyloliquefaciens* AS385 [30], *Bacillus methylotrophicus* K26 [31], *Bacillus subtilis* B2 [32], and *Paenibacillus* sp. TKU042 [33] also possess the α-GI-producing ability to a large extent. Taken together, it can be stated that *Bacillus* species is a predominant microorganism that can be potentially used to yield α-GIs and help to regulate postprandial hyperglycemic pathophysiology associated with diabetes. *Bacillus* species stand out for their dual functionality, combining robust probiotic attributes with exceptional fermentation characteristics [24]. Notably, strains such as *B. subtilis* exhibit resilience in gastrointestinal conditions, making them effective probiotics that contribute to gut health and immune modulation. Simultaneously, their impressive fermentation capabilities enable the efficient conversion of various substrates into valuable products like enzymes and bioactive compounds that have tremendous health and therapeutic benefits.

In this investigation, a total of 23 bacteria were isolated from peruyaan, a traditional fermented soy food of Arunachal Pradesh. Among these diverse bacterial colonies, four remarkable strains (P2 MEK, P4 MEK, P6 MEK, and P8 MEK) were recognized as α-GI producers (Figure 1). Moreover, from these four strains, the most potent strain has been characterized, identified, and designated as *Bacillus subtilis* PM_NEIST_4 (Figure 2). Thereafter, the study focuses on optimizing the fermentation process using *B. subtilis* PM_NEIST_4 by exploring cultivation parameters such as culture media, temperature, culture volume, and OD_{600} of bacteria in media [28] (Figures 3 and 4).

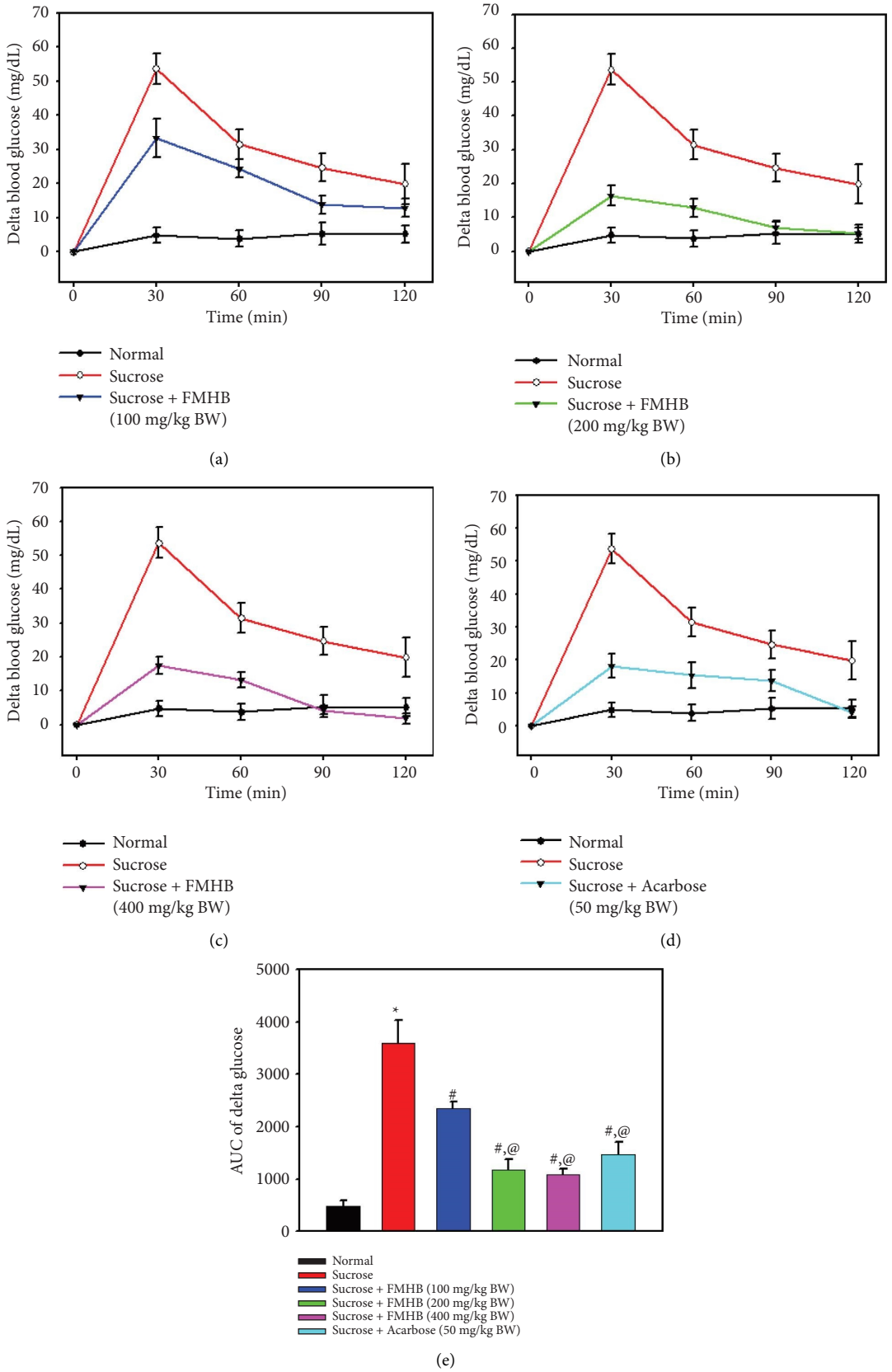

Figure 8: The effect of FMHB (a) 100 mg/kg·BW; (b) 200 mg/kg·BW; (c) 400 mg/kg·BW); and acarbose (d) on postprandial plasma glucose level in sucrose fed male Wistar rats. Blood glucose level was measured at a regular interval of 30 min consecutively for 2 h. The areas under the curve (AUC) of the glucose response was analysed (e). Data are expressed as mean ± SD (n = 6). "∗" denotes the significant difference from normal (received water) ($p < 0.05$); "#" denotes the significant difference from sucrose-fed group ($p < 0.05$); @ denotes the significant difference from FMHB treated group at a dose of 100 mg/kg BW ($p < 0.05$).

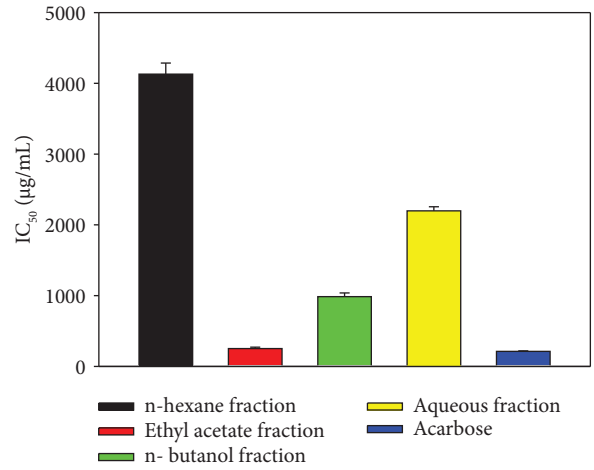

FIGURE 9: A comparative study between the IC_{50} values of different solvent fractions extracted from FMHB with standard, acarbose. Data are expressed as mean ± SD ($n = 3$).

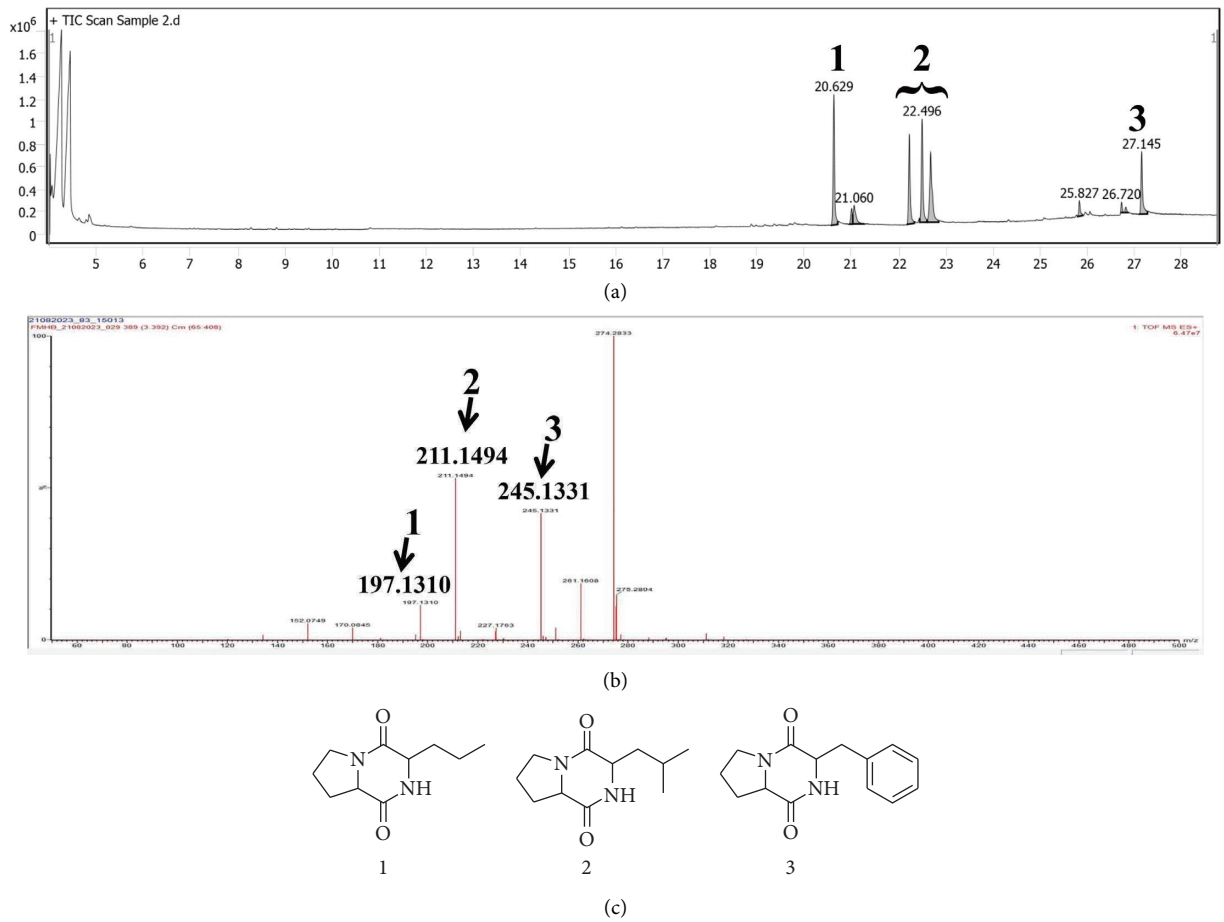

FIGURE 10: GC-MS chromatogram (a) and LC-MS chromatogram (b) of ethyl acetate fraction of FMHB. Both GC-MS and LC-MS analyses revealed the presence of three bioactive compounds- compound 1 (3-propylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione), compound 2 (3-isobutylhexahydropyrrolo [1,2-a]pyrazine-1,4-dione), and compound 3 (3- benzylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione) (c).

Optimization of these culture parameters led to a significant increase in α-GI productivity, reaching 4808.324 ± 137.26 U/mL. In comparison, before optimization, the productivity was 1616.613 ± 84.54 U/mL (Table 1). Furthermore, an initial IC_{50} value of 1705.617 ± 43.95 μg/mL was improved to a value of 596.532 ± 44.80 μg/mL (Figure 5). Both these α-GI productivity and IC_{50} resulted after optimization strongly supported that the cultivation parameters considered to yield maximum α-GI productivity were successfully able to improvise the FMHB by approximately three times.

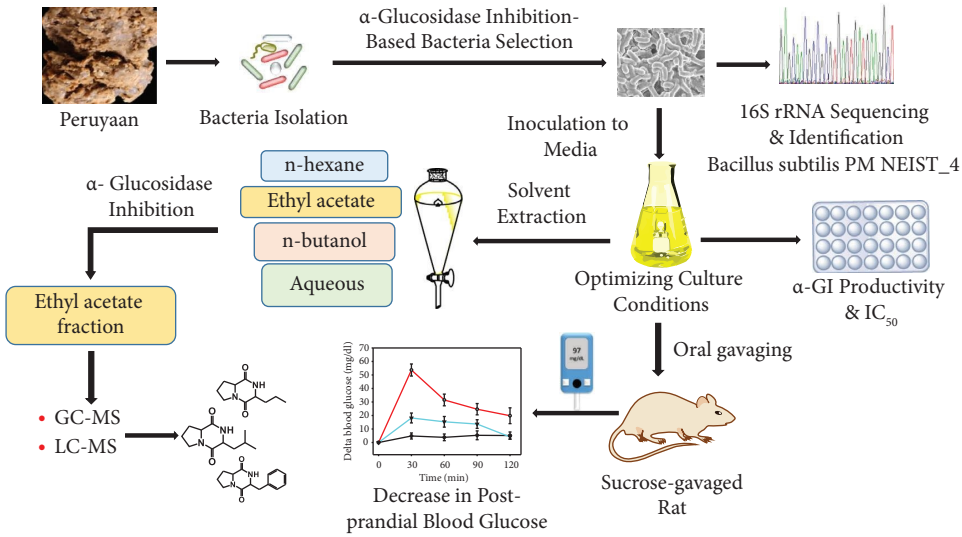

Figure 11: Schematic presentation illustrating the isolation of α-glucosidase inhibitor (α-GI) producing bacterial strain from peruyaan, a traditional fermented soy food of Arunachal Pradesh, India. The workflow involves steps to optimize the production of α-GI for maximum yield, validation in animal model, and assess the compounds produced by the bacterial strain.

The α-GI produced by *B. subtilis* PM_NEIST_4 was also proved to have high thermal and pH stabilities up to 120°C and pH 13, respectively, with more than 90% activity observed in both the cases (Figure 6). This signifies that the α-glucosidase inhibitory property of the optimized FMHB would remain unaffected even when exposed to high temperature. Thus, it may be concluded that the FMHB can persist and maintain its stability for a longer duration. At the same time, as the pH of the gastrointestinal tract varies greatly, therefore it is one of the essential factors that must be considered for determining the pH stability of α-GI produced in the culture supernatant of FMHB. In this regard, the experimental results indicate that the FMHB can withstand various pH levels in the digestive tract without compromising its α-glucosidase inhibitory activity.

Treatment with FMHB demonstrated no toxic effects in both cellular and animal models, even at higher concentrations (Figure 7, Tables 2, and 3). For elucidating the effect of FMHB on animal model, three different doses of FMHB were administered to the sucrose-fed rat model. Results demonstrated that two doses, namely, 200 mg/kg and 400 mg/kg showed remarkable reduction in postprandial plasma glucose level at different time intervals, almost comparable to that of positive control (acarbose) (Figure 8). The results indicate that FMHB has the potential to contribute to lowering postprandial blood glucose by inhibiting the breakdown of sucrose into glucose in the intestinal tract. Thereafter, FMHB was fractionated using solvents of increasing polarity that include n-hexane, ethyl acetate, and n-butanol. The ethyl acetate fraction of FMHB showed the highest inhibitory activity against α-glucosidase enzyme (Figure 9). Based on this result, the ethyl acetate fraction was allowed to undergo GC-MS and LC-MS analyses for compound assessment. In this regard, GC-MS analysis of the ethyl acetate fraction demonstrated five major peaks at different acquisition times and LC-MS analysis of the same also revealed the presence of different compounds based on calculated mass. Both the analyses of ethyl acetate fraction suggested the presence of three pyrrolopyrazine derivatives-3-propylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione, 3-isobutylhexahydropyrrolo [1,2-a]pyrazine-1,4-dione, and 3-benzylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione (Figure 10). Pyrrolopyrazine derivatives have been reported to exhibit a wide range of biological activities including antioxidant, anti-inflammatory, kinase inhibitory, antimicrobial, antiviral, antifungal, as well as antitumor [52]. However, there is no report so far that advocates the α-glucosidase inhibitory efficacy of pyrrolopyrazine and its derivatives.

The present study demonstrated the isolation of a potent α-GI producing bacterial strain, *B. subtilis* PM_NEIST_4 from traditional fermented soy food (peruyaan), and thereafter, the optimization of bacterial fermentation process was achieved to maximize the yield of α-GI (Figure 11). These findings underscore promising industrial potential of *B. subtilis* PM_NEIST_4, advocating the use of indigenous microbial resources for the production of α-GI.

5. Conclusion

Numerous commercially available α-GIs have been used clinically to manage hyperglycemia in diabetic conditions. Despite their effectiveness, these drugs are often associated with undesirable side effects, including diarrhea, nausea, vomiting, kidney problems, and liver damage. In addition, a significant disparity exists between the substantial production of α-GIs in the industry and their extensive consumption in the market. To address this challenge, there is a need to explore safer natural α-GIs and maximize its productivity in order to meet the ends. In this study, *Bacillus*

subtilis PM_NEIST_4, isolated from peruyaan, showed remarkable inhibition of α-glucosidase enzyme activity. In order to enhance the productivity of α-GI, fermentation conditions have been optimized. This imperative optimization process aims to fix various parameters within the fermentation environment, including C/N source, cultivation temperature, medium/system volume ratio, OD_{600} of *B. subtilis* PM_NEIST_4 in culture media, and cultivation duration, with the overarching goal of maximizing the yield of α-GIs. This finding not only emphasizes the significant role of this bacterium for production of α-GI but also suggests its promising application as a therapeutic tool for managing hyperglycemia. While the results showed promising evidence of its antidiabetic potential, it is however necessary to perform further studies in order to fully understand the underlying mechanism of its action. Hence, clinical trials are encouraged for evaluating the antihyperglycemic efficacy of α-GI produced by this bacterium. In conclusion, the outcome of this study will be helpful for the development of a novel therapeutic approach to prevent the attainment of high blood glucose concentrations in diabetic conditions. This has significant implications for the effective management of hyperglycemic pathophysiology in individuals with diabetes.

Abbreviations

α-GI: α-Glucosidase inhibitor
BHI: Brain Heart Infusion
FBS: Fetal bovine serum
FMHB: Fermented Mueller Hinton Broth
GC-MS: Gas chromatography-mass spectrometry
LC-MS: Liquid chromatography-mass spectrometry
LB: Luria Bertani Broth
NA: Nutrient Agar
NB: Nutrient Broth
OD: Optical density
PBS: Phosphate-Buffered Saline
SEM: Scanning electron microscopy
TSB: Tryptone Soya Broth.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Ethical Approval

The animal study was approved by the Institutional Ethical Committee and CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals), Ministry of Environment and Forests, New Delhi, India (2028/G)ReBi/S/18/CPCSEA).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Mir Ekbal Kabir conceptualized the study, performed investigation, proposed the methodology, contributed to data curation, perform formal analysis, wrote the original draft, wrote, reviewed, and edited the article, and contributed to visualization. Anupriya Borah performed investigation, proposed the methodology, contributed to data curation, performed formal analysis, wrote the original draft, and reviewed and edited the article. Hiranmoy Barman, Nazim Uddin Afzal, and Bhaben Sharmah performed investigation, contributed to data curation, and reviewed and edited the article. Tridip Phukan performed formal analysis, supervision, and validation. Jatin Kalita performed supervision and validation. Prasenjit Manna conceptualized the study, performed investigation, proposed the methodology, contributed to data curation, performed formal analysis, provided resources, performed supervision, prepared the original draft, reviewed and edited the article, and performed validation. Mir Ekbal Kabir and Anupriya Borah contributed equally to this manuscript.

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