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Qudsia Mushtaq, Uzair Ishtiaq, Nicolas Joly, Alexis Spalletta, Patrick Martin. Harnessing *Bacillus subtilis* QY5 PP784163 for Bioethanol Production from Potato Peel Waste and Nutrient Recovery for Animal Feed: Maximizing Resource Efficiency. *Fermentation*, 2024, <10.3390/fermentation10100523>. <hal-04735025>

HAL Id: hal-04735025

<https://hal.science/hal-04735025v1>

Submitted on 14 Oct 2024

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Harnessing *Bacillus subtilis* QY5 PP784163 for Bioethanol Production from Potato Peel Waste and Nutrient Recovery for Animal Feed: Maximizing Resource Efficiency

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Abstract: The present work focuses on the utilization of potato peel waste for the production of bioethanol. In the present study, extensive screening was undertaken to isolate amyolytic and cellulolytic microbes using starchy biomass. After confirming the chemical composition of potato peel waste (PPW), several trials were performed to enhance the amylase and cellulase production from *Bacillus subtilis* to hydrolyze the PPW in submerged fermentation. Optimization of physical parameters was performed using both commercial and indigenous media from enzymatically hydrolyzed PPW. Different routes of various combinations were designed to enhance bioethanol production. The maximum ethanol titer of 0.50% and 0.41% was recorded in Route B and A, i.e., separate saccharification and ethanol fermentation and consolidated fermentation. Simultaneous saccharification and fermentation (SSF) also measured a good ethanol yield of 0.46%. The fermented residual cake was checked for nutritional components and showed a high content of protein and amino acids because of the addition of unicellular yeasts. This cake can be utilized as an animal feed supplement.

Keywords: potato peel waste; *Bacillus subtilis*; consolidated bioprocessing; *Saccharomyces cerevisiae*



Citation: Mushtaq, Q.; Ishtiaq, U.; Joly, N.; Spalletta, A.; Martin, P. Harnessing *Bacillus subtilis* QY5 PP784163 for Bioethanol Production from Potato Peel Waste and Nutrient Recovery for Animal Feed: Maximizing Resource Efficiency. *Fermentation* **2024**, *10*, 523. <https://doi.org/10.3390/fermentation10100523>

Academic Editors: Mohamed Koubaa and Hironaga Akita

Received: 24 August 2024

Revised: 5 October 2024

Accepted: 9 October 2024

Published: 14 October 2024



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1. Introduction

Approximately one-third of globally produced food is estimated to be wasted along the food chain, contributing to the environmental burden and inefficiency of the food system [1,2]. Reducing food waste along the supply chain can contribute positively towards sustainable development goals, but the differences in food waste in terms of nutrients and environmental impacts are not well-known [3]. For many years, agricultural waste and domestic garbage, encompassing vegetables and fruit skins, were not properly disposed of, causing many side effects [4–7]. The improper practice of dumping and burning agricultural waste on open land is still observed in many developing countries, which has resulted in significant hazards to the environmental health and biota [8–12]. Followed by environmental contamination, these conventional practices of waste dumping also caused heavy economic losses that cannot be ignored [13]. Such locally produced agricultural food wastes should be used sustainably for the production of valuable goods. The waste biomass can be used as an attractive feedstock for clean fuels, which are workable alternatives for future energy [14]. The global food waste production, approximately 1.3 billion tons annually, is equivalent to one-third of the food made for human consumption [15]. According to the Food Waste Index Report, nearly 931 million tons of food waste, including

household, food service and retail waste, were produced in 2019 [16]. Biotechnological approaches are used as innovative and green applications to valorize agricultural food wastes and improve their stability and applicability.

Currently, 80% of the demand for energy at the global level is accomplished by fossil fuels Klemsa [17] and Sharma [18]. In response to this situation, there has been a continuous rise in research and development for biofuels on a commercial scale Kularathne [19] and Nascimento [20]. Alternative to petroleum sources, biofuels can be used as an ideal fuel, thus becoming dominant in the transportation sector Mostofa [21], Susmozas [22], Lamichhane [23], Edeh [24] and Ma [25].

Around the world, potato is the fourth most abundant vegetable crop. According to “FAO United Nations”, potato production has reached 376 million metric tons globally in 2021 Singh [26], FAO [27] and Mickiewicz [28]. According to the International Potato Centre (CIP), the global consumption of potatoes is shifting from fresh potatoes to value-added food products. Due to the excessive use of potatoes in various sectors for the production of products on a commercial scale, a vast amount of potato peel waste is produced during processing, such as peeling, trimming and cutting, which is unavoidable Liang [29] and Pathak [30]. This residual material encompasses 15–40% of potato weight, depending on the abrasive, steam or lye peeling process Schieber [31] and Barampouti [32]. Landfilling is the most widely used management practice for PPW, liberating greenhouse gases and leachates that corrode environmental health Chohan [33] and Ebrahimian [34]. PPW is highly prone to microbial deterioration due to moisture content, leading to a limited lifespan. The current trend towards the valorization of wastes demands the conversion of industrial waste into value-added products Wu [35].

PPW is a lignocellulosic substrate that comprises lignin, cellulose, hemicellulose and starch. PPW comprises phenolic acids and polyphenols known for their antioxidant potential, whereas fatty acids and lipids are responsible for antimicrobial activities. PPW also contains starch and non-starch polysaccharides, lignin, and ash Maldonado [36], Jeddou [37] and Liang [29]. Due to its nutritional status, this waste can be considered a promising feedstock for various industrial sectors. The potential of this waste has been exploited in various sectors, such as the food industry, farming, medicine and energy. Valuable compounds such as phenols, alkaloids and free-radical scavenger abilities have drawn the attention of various industries that are seeking natural sources for the production of chemicals Wu [38], Hussain [39], Galhano [40], Mushtaq [41] and Mushtaq [42].

Fermentation technology is broadly used for the production of different important compounds that have applications in the energy production, drug, chemical and food industries. In spite of the fact that fermentation processes have been utilized for many years, the requirement for sustainable creation of items to meet the market prerequisites in a cost-effective way has put forward a difficult challenge. For any fermentation-based product, the priority is the accessibility of fermented products that are equal to market demand. Different microorganisms have been reported to produce an array of primary and secondary metabolites but in a very low quantity. To satisfy the market need, a few high-yielding procedures have previously been found and effectively implemented in various processes like biotransformation, oil extraction and production of primary and secondary metabolites Dubey [43], Dubey [44], Singh [45] and Rajeswari [46].

PPW processing can be carried out by biological and thermochemical processes. In biological processing, microorganisms are used to convert PPW into bioproducts through fermentation Abedini [47], whereas, in thermochemical processing, PPW is transformed into bioproducts by thermal decomposition and pyrolysis Diamary [48]. Both these processes have multiple challenges and still require auxiliary processes or technologies to enhance the hydrolysis of substrate. In past years, various methods have been extensively investigated to enhance bioethanol production using raw substrate. In consolidated bioprocessing, three important steps, liquefaction, hydrolysis and fermentation, are performed by fermenting yeast for conversion of starch into bioethanol Van Zyl [49]. For the past few years, much work has been conducted to reduce the cost of cellulase enzymes. Consoli-

dated bioprocessing (CBP) offers higher process efficiency and lower cost than the processes presenting cellulase production. Still, it has been a challenge to run all biological processes in a single container with substrate utilization and product formation Lynd [50]. Liu et al. recorded an ethanol yield of 0.68 g/L performing CBP using *Clostridium thermocellum* DSM 1237 from sugarcane bagasse (SCB) [51]. CBP can increase the production of biofuel and other useful products, which can compensate for the additional cost of the process. In our study, we first checked separately the potential of yeast to convert starch-containing substrate into ethanol. Later, we checked the combination of both bacterial and yeast strains and whether this combination is more productive than alone yeast. Separate and simultaneous hydrolysis and fermentation have been proven as effective strategies to optimize the conditions of both enzymatic degradation and hydrolysis Malsova [52]. Bacterial saccharification and fermentation for the production of bioethanol have been extensively investigated [52]. PPW can be effectively used for the production of animal feed and fertilizer and as raw material for biogas. Excessive nutritive materials present within peels with antioxidant, antibacterial and anti-inflammatory properties can be effectively utilized. Fermented PPW is an excellent source of nitrogen for ruminants. Using potato-based protein as an additive for livestock feed has been an old-age practice. Potato residue can effectively replace barley as an energy source for beef cattle and other livestock Kannuchamy [53].

In the present study, these different routes were practically designed and performed to check the potential of PPW for bioethanol production using submerged fermentation. Amylolytic and cellulolytic bacterial strain *Bacillus subtilis* QY5 PP784163 was isolated from the local environment and used as a source of crude enzymes that degrade various highly packed polymers and make their conversion easy into bioethanol. Commercially available baker's yeast was coupled with bacterial strain to enhance bioethanol production. Various physical parameters such as temperature, pH, inoculum size and incubation time were optimized for both commercial and indigenous media using submerged fermentation. The residual fermentation cake that remained after the experiment enriched with yeast containing high protein and amino acid content can be utilized as an animal feed supplement.

2. Materials and Methods

All steps of the current study are described in Figure 1, including isolation and selection of microbial agents, preparation of indigenous medium, and sequence of different steps taken to optimize bioethanol yield.

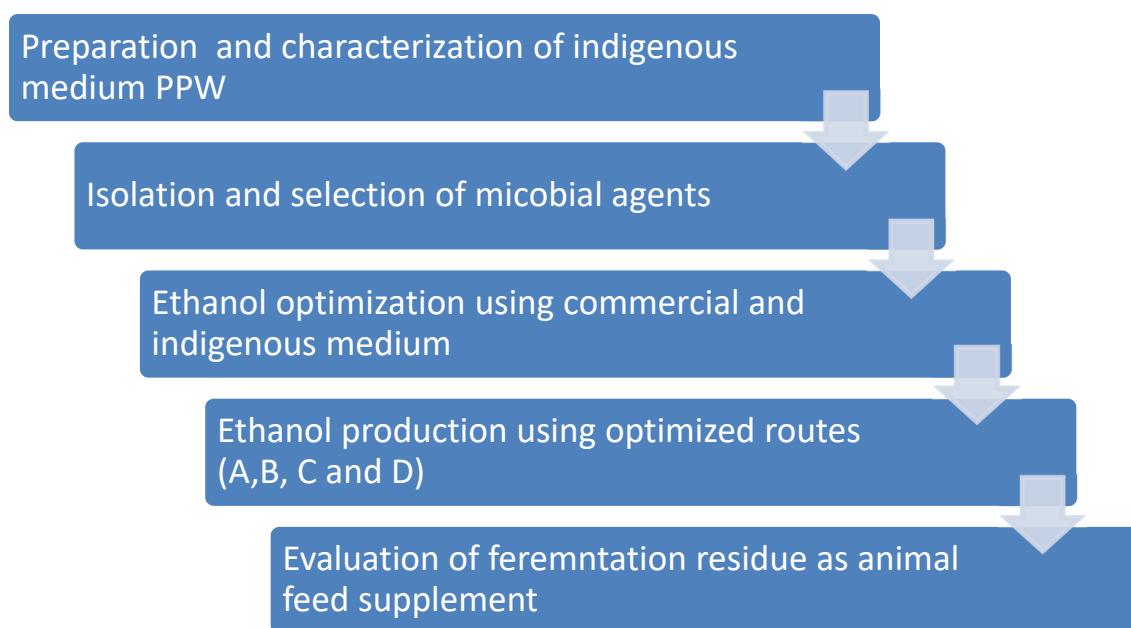


Figure 1. Flow sheet diagram representing all steps carried out to optimize ethanol production.

2.1. Substrate Preparation and Characterization

Potato peel waste was taken from a local fries shop situated at Quaid-e-Azam campus, Gate no.4 of the University of the Punjab, Lahore, Pakistan. Peels were washed properly to remove dust, dried in sunlight for 24 h, and then oven-dried at 60 °C for 72 h until a constant weight was reached. Using an electric mill, peels were powdered in order to obtain a uniform size of 2 mm. The pulverized peels were stored in airtight containers at room temperature 25 °C (± 5 °C) until further use. The content of various compounds, including carbohydrates, protein, fat, fiber, ash and moisture, was determined in raw PPW by following the protocols of AOAC [54].

2.2. Yeast Sample

Saccharomyces cerevisiae was isolated from commercial baker's yeast, which is produced by Guangxi Sungain Yeast Technology Co., Ltd., Nanning, China. A slant of PDA (Potato Dextrose Agar) was used to preserve the sample. The yeast sample was revived in freshly prepared selective medium yeast peptone extract (YPD), which contained the following components: 2% glucose (anhydrous), 2% peptone and 1% yeast extract. The active fresh yeast culture was used as inoculum for submerged fermentation. For further use, the yeast culture was preserved at 4 °C. Indigenous media used for the cultivation of the yeast sample contained PPW 50 g/L and yeast extract 10 g/L. YPD and PPW are both extensively used media for the growth of various microbial strains.

2.3. Isolation of Bacterial Strains

Different lab-isolated bacterial strains were collected from waste and the discarded and spoiled fruits of a vendor shop located near the University of Punjab, Lahore, Pakistan. Spoiled fruits were chosen as isolating material because microbes residing on fruits produce high titer of exoenzymes, i.e., amylases and cellulases. Sampling material was taken in sterilized vials and further reached in labs for processing. Temperature and pH of the original place were measured. The pure culture was obtained by cross streaking on nutrient agar plates and followed by repeated streaking. This procedure was performed till pure culture was maintained. The whole isolation procedure was performed in a clean Microbial Biotechnology lab near a hot flame where sterile air was present. Ultraviolet lamps were turned on in a laminar flow hood for 15 min to ensure the removal of any microbial growth.

2.4. Culture Media and Isolation of Mesophilic Bacteria

The samples of different bacterial strains were serially diluted and then inoculated on nutrient agar (Peptone 5 g, yeast extract 1.5 g, beef extract 1.5 g sodium chloride 5 g, agar 15 g/L, pH 7.4 \pm 0.2 at 25 °C). Different colonies were selected from general-purpose medium nutrient agar. Isolated colonies were further grown on the indigenous selective medium. All the cultures were raised at 37 °C for 48 h to 72 h. The selective medium for screening of amylolytic and cellulolytic bacteria comprised of K₂HPO₄, KH₂PO₄, NaCl, MgSO₄·7H₂O, (NH₄)₂SO₄, yeast extract, agar, starch (for amylase)/carboxy methyl cellulose (CMC) for cellulase [55]. Self-constructed media comprised of 5% PPW and 1% yeast extract was also used for the production of crude enzymes, i.e., amylase and cellulase. To check the pH tolerance of isolated bacteria, the sterile nutrient broth was prepared with different pH such as 6, 6.5, 7.0, 7.5, 8.0, and after inoculation, they were incubated at 37 °C for 72–96 h. To check thermostability, the culture temperature was maintained at various values such as 37, 40, 50 and 60 °C with shaking maintained at 150 rpm for 72–96 h. The growth of bacteria was checked at 600 nm using a light spectrophotometer. To preserve bacteria, glycerol stocks were prepared with bacterial culture and preserved at –40 °C.

DNA Extraction and taxonomic identification of bacterial isolates: To extract genomic DNA, 3 mL of incubated culture was centrifuged, the resulting pellet was suspended in lysis solution, and the supernatant was stored in Eppendorf tubes, which were subsequently stored at –40 °C until further use. To assess the integrity of DNA, electrophoresis was performed using 0.5% agarose gel containing EtBr in 0.5 \times TAE buffer. The bacterium

was isolated by repeatedly streaking on nutrient agar medium and stored at 4 °C. It was identified by 16S rRNA gene sequencing, and a detailed protocol of molecular identification has been described in an earlier report [56]. The nucleotide sequence was compared using the BLAST method of NCBI. Molecular evolutionary and genetic analysis MEGA X software version 5.01 was used to align the sequence for *Claustral W*. The phylogenetic tree of isolated bacterial strain QY5 PP784163 was constructed by following the neighbor-joining method with bootstrap values of 500 replications, as shown in Figure 4.

2.5. Selected Mesophilic and Amylolytic Bacterium Used for Fermentation

Preliminary experiments were carried out for each bacterial strain, and the strain that showed maximum amylase and ethanol production was used in optimized routes of ethanol production. The amount of indigenously produced amylase and cellulase was measured by following the protocols of [57,58].

2.6. Media Used for Fermentation

Both commercial and indigenous media were utilized to optimize ethanol production. Media comprising 5% PPW and 1% yeast extract with pH 6 was used as feedstock for enzyme production. After sterilizing media, it was inoculated with 2% inoculum of freshly prepared *B. subtilis* QY5 PP784163 and incubated at 37 °C for 72 h on 120 rpm. After 72 h of fermentation, samples were taken out and centrifuged at 4 °C and 10,000 rpm for 10–15 min. After removing the bacterial pellet, a clear supernatant was examined for amylase and cellulase assay.

2.7. Ethanol and Glucose Tolerating Potential of Selected Strains

B. subtilis and *S. cerevisiae* were further tested for glucose and ethanol tolerance. Dissimilar concentrations of glucose and ethanol, i.e., 5, 10, 15 and 20%, were added to sterilized YPD media. The media-holding reactors were inoculated with 2% freshly prepared inoculum of *S. cerevisiae* and incubated at 35 °C for 120 h. Afterward, yeast growth was measured at 600 nm. To check bacterial tolerance, different concentrations of glucose and ethanol were prepared in a sterilized medium of nutrient broth and incubated for two days. The growth was read at 600 nm.

2.8. Ethanol Fermentation

Using a 250 mL Erlenmeyer flask, the experiment was carried out in triplicates. Sterilized commercial medium of YPD and self-constructed indigenous medium PPW was prepared and autoclaved, cooled, and inoculated with 2% *w/v* growth of 24 h old culture of *S. cerevisiae*. After inoculation, flasks were maintained at 150 rpm for 48 h at 37 °C, and growth was observed phenotypically. A two-inch-thick layer of sterilized paraffin oil was poured for the anaerobic condition and maintained by a tightly closed lid of the flask for five days. After five days, samples were centrifuged at 10,000 rpm, and supernatant free of microbial growth was collected in the test tube to determine ethanol content using the potassium dichromate test. Fermentation was preliminarily checked each day for three to seven days, and optimum results were obtained after four days; yeast growth also takes three days to grow, and afterward, the fermentation phase usually flourishes. That is why five days were selected as the optimum time period.

2.9. Optimization of Ethanol Yield Using Commercial and Indigenous Medium

The *S. cerevisiae* was grown in commercially available YPD and self-constructed media. Physical factors, including temperature and pH, were selected as 30, 35, 37, 40 °C and 4, 5, 5.5, 6.5 pH, respectively. Inoculum of 2, 4, 6 and 8% and time of incubation of 2, 4, 6 and 8 days were taken to optimize ethanol production, respectively. Following the yeast growth and fermentation, ethanol content was determined using a potassium dichromate test.

2.10. Ethanol Content Estimation Using Acid Dichromate Test

With the help of a dichromate test, the ethanol content of fermented samples was evaluated [59]. The following equation was used to determine the ethanol quantity:

$$\text{Ethanol (mg/mL)} = \text{Curve value} \times \text{Dilution factor} \times 2$$

2.11. Statistical Analysis

All experiments performed in triplicates were depicted as average \pm s.e.m. Analysis of variance (ANOVA) with $p \leq 0.05$ was used to analyze the data using Minitab (16th version).

2.12. Co-Culturing of Yeast and Bacterial Strain

The compatibility of both microbial strains, i.e., *B. subtilis* and *S. cerevisiae*, was checked visually by a consortium. To develop a consortium, newly isolated *B. subtilis* QY5 PP784163 of 100 μ L was spread in the form of lawn using a spreader on sterilized nutrient agar plates. After drying this bacterial lawn, filter paper discs impregnated with 100 μ L of *S. cerevisiae* were placed carefully at equal distances in laminar flow. Following incubation of 72 h at 37 °C, bacterial growth was not inhibited around the filter paper discs comprising yeast culture. This growth around the edges of filter paper discs confirmed the compatibility of both microbial species.

2.13. Ethanol Production Using Various Routes

The commercially available *S. cerevisiae* was used to produce ethanol using different routes, as depicted in Figure 2. Self-constructed media contained 50 g/L PPW, and yeast extract 10 g/L was used in all the routes. Different experimental conditions were maintained in each route, and following fermentation, samples were centrifuged, and growth-free supernatant was used to determine ethanol yield using HPLC. The left-over fermentation cake comprising bacterial and yeast growth and starch-rich PPW was analyzed by proximate analysis to check the content of various useful compounds such as carbohydrate, protein, nitrogen, fiber and moisture.

2.13.1. Consolidated Fermentation/Route A

In this route, a freshly prepared culture of 2% *w/v* of both microbial strains, i.e., *B. subtilis* QY5 PP784163 and *S. cerevisiae*, was co-cultured and allowed to ferment in Erlenmeyer flasks for five days. Sterilized paraffin oil of a four-inch-thick layer was poured to maintain anaerobic conditions and increase ethanol production. The experimental flasks were incubated for five days at 37 °C. Afterward, samples were taken out to analyze ethanol content.

2.13.2. Separate Saccharification and Ethanol Fermentation/Route B

In Route B, a freshly prepared culture of 2% *w/v* of *B. subtilis* was allowed to ferment PPW for 48 h. Afterward, the whole media was centrifuged for 10–15 min at 10,000 rpm. Clear supernatant free of any bacterial growth was filtered using a Millipore filter of 0.45 μ m size to eliminate any bacterial growth. This filtered culture fluid was inoculated with 2% *w/v* of freshly prepared yeast culture. A thick layer of sterilized paraffin oil was poured to maintain anaerobic conditions, and lids were tightly closed and allowed to incubate for five days at 37 °C. After completion of the specific time period, samples were taken out to analyze ethanol yield.

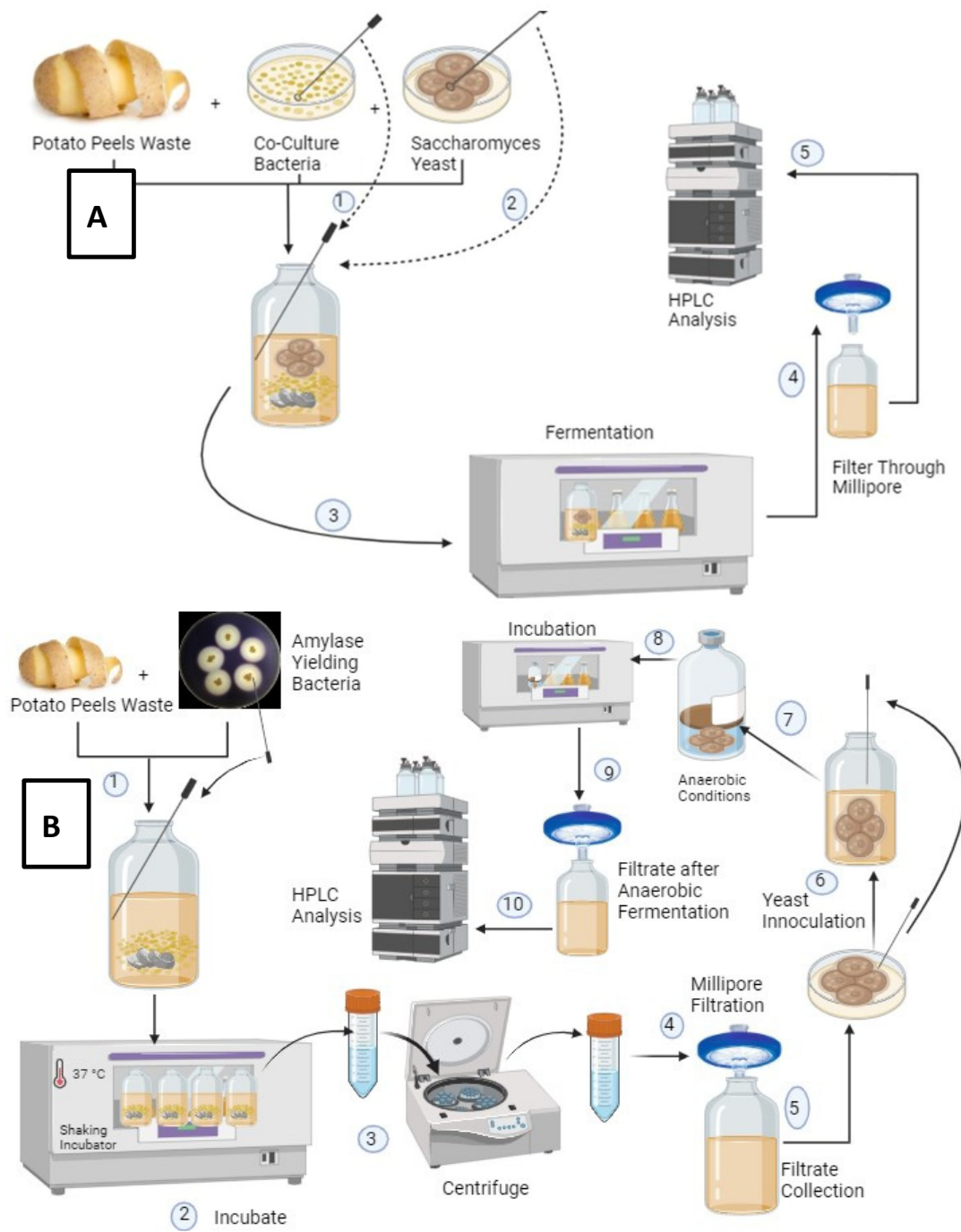


Figure 2. Cont.

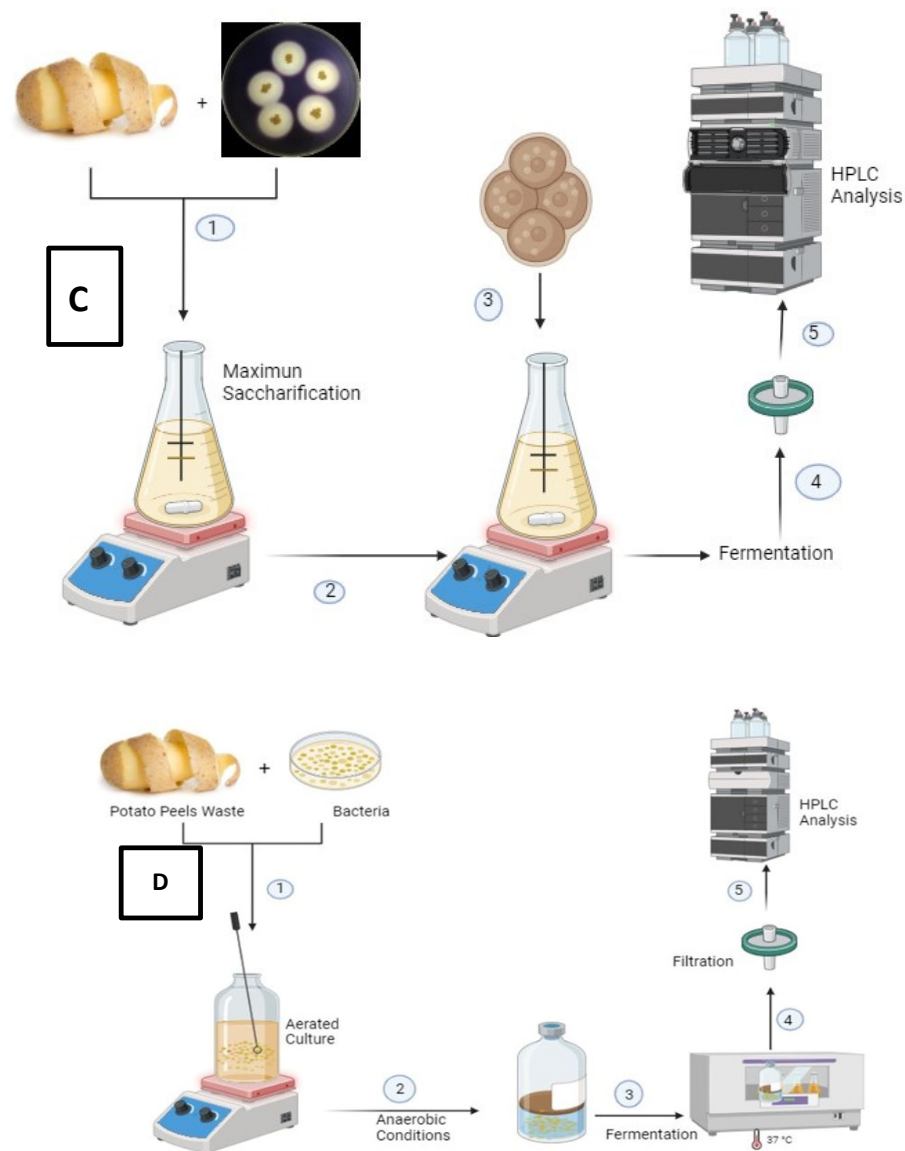


Figure 2. Different routes of ethanol production: (A) consolidated bioprocessing; (B) separate saccharification and fermentation; (C) two-step saccharification plus fermentation; (D) bacterial saccharification and fermentation.

2.13.3. Two-Step Saccharification Plus Ethanol Fermentation/Route C

In Route C, a freshly prepared culture of 2% *w/v* of *B. subtilis* was inoculated to ferment substrate for 72 h. Maximum degradation of starch into small monomeric units was performed. After 72 h, a freshly prepared culture of *S. cerevisiae* of 2% *w/v* was inoculated to this media comprising bacterial culture. *S. cerevisiae* inoculation was followed by *B. subtilis* inoculation because saccharification was the first step required to break complex substrates utilizing enzymes. Eventually, the degraded substrate was fermented by yeast culture. Sterilized paraffin oil was used to maintain anaerobic conditions, and the lids were tightly closed and incubated for five days at 37 °C. Afterward, samples were taken out to analyze ethanol yield.

2.13.4. Bacterial Saccharification and Fermentation/Route D

In this route, conditions were maintained to perform bacterial saccharification and fermentation. In the initial step, a freshly prepared culture of 2% *w/v* of *B. subtilis* QY5 PP784163 was used for saccharification of PPW for 48 h using aerobic conditions. After 48 h,

conditions were shifted towards fermentation by following anaerobic conditions. Tightly closed flasks were incubated for five days at 37 °C. Afterward, samples were analyzed to determine ethanol yield.

2.14. Ethanol Quantification by HPLC

RP-HPLC Perkin Elmer, Waltham, MA, USA, using Zorbax C18 column determined ethanol production. A mobile phase of 0.005 M sulphuric acid with column temperature 60 °C and flow rate of 0.6 mL/min was maintained. A sterile filter of 0.2 µm and an injection volume of 20 µL was used to filter the sample. With the help of a standard curve, ethanol concentration was calculated. The ethanol estimation of optimized routes, including A, B, C and D, was performed by HPLC.

2.15. Determination of Nutritional Status of Fermentation Residue by Proximate Analysis

The residue left behind after the completion of fermentation routes was analyzed by proximate analysis to determine the content of various useful compounds. PPW was characterized by following the protocols of AOAC [54]. The content of various compounds, including carbohydrates, protein, fat, fiber, ash and moisture, was determined. PPW's nutritional status was checked before and after fermentation to determine its potential as an animal feed supplement.

3. Results

In this study, different bacterial species were isolated from discarded and spoiled fruit samples. The temperature and pH of the sampling site were recorded as 40–45 °C and 6.5–8.5, respectively. After obtaining pure culture and identification, these species were used in various preliminary experiments where different factors like amylase and cellulase production and ethanol production were optimized. The bacterial strain that produces a high concentration of lucrative enzymes and confirmed compatibility with baker's yeast was selected to be used in an optimized experiment of ethanol production. *B. subtilis* QY5 was selected to be used in fermentation experiments, and its amylase and cellulase-producing potential was estimated to be 0.5742 and 1.1911 IU/mL/min, respectively. Coupling of *B. subtilis* QY5 was confirmed with commercially available baker's yeast by cross streaking, and different routes were designed to optimize bioethanol production using enzymatically hydrolyzed PPW. The phenotypic representation of both selected strains, *B. subtilis* and *S. cerevisiae*, and their amylolytic potential is shown in Figure 3.

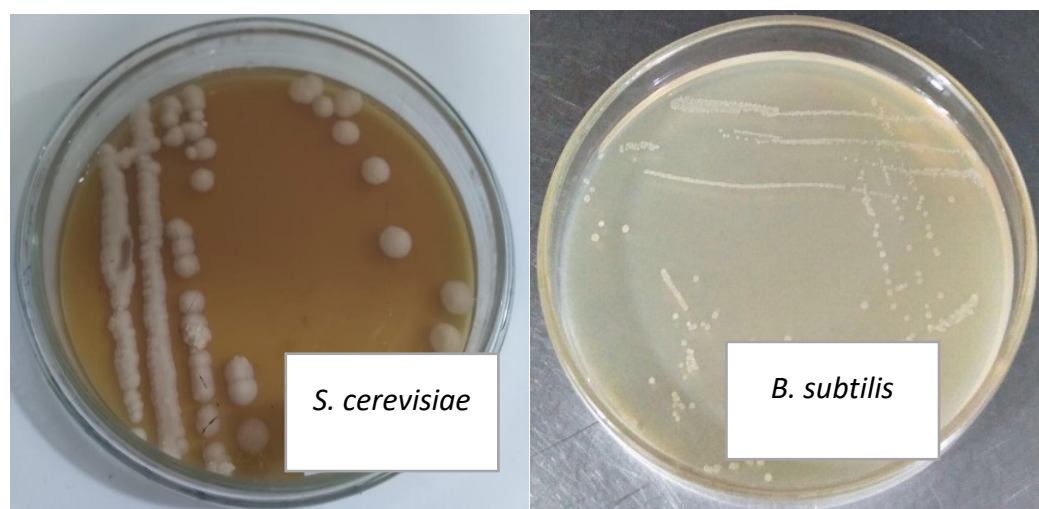


Figure 3. Cont.

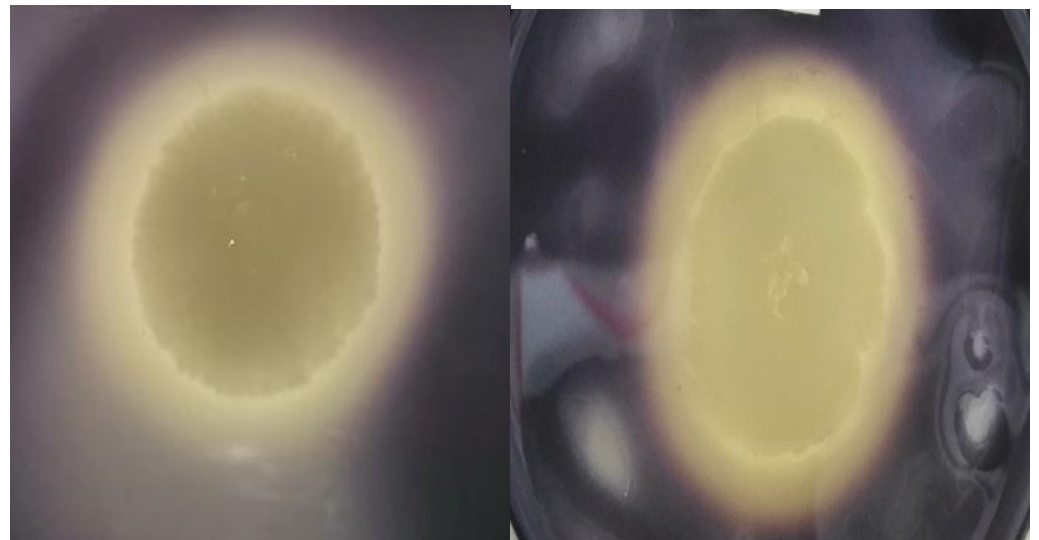


Figure 3. Phenotypic appearance and amyolytic zone depiction of selected strains on selective medium after iodine flooding.

3.1. Morphological Characteristics of *Bacillus subtilis* QY5 PP784163

A total of twenty bacterial strains were isolated, and further screening was performed on the basis of morphological differences. Amylase- and cellulase-producing potential was confirmed on selective media comprising starch and CMC. The *Bacillus subtilis* QY5 PP784163 was isolated on nutrient agar, and its potential was also checked in an indigenous medium. *B. subtilis* QY5 PP784163 NR_104873.1 was grown on nutrient agar plates, incubated at 37 °C for 2–3 days, maintained at 4 °C and sub-cultured at four-week intervals.

The growth patterns of the colony showed diversity in response to various environmental factors, including nutrient and agar concentration. The colony was observed as creamy white in color and medium in size with circular margins. The colony size was variable in response to incubation time; the colony size of 8–12 mm of *Bacillus subtilis* QY5 PP784163 NR_104873.1 was recorded after 72–96 h of incubation at 37 °C on nutrient agar media. The thermos-stability of the isolated strain was checked at various temperatures such as 30, 40, 50 and 55 °C; optimum growth was observed within a temperature range of 30–40 °C. This classifies the isolate as a mesophilic bacterial species. The pH range for maximum proliferation was found to be between 6 and 8. Microscopic observations revealed that the isolated strain corresponded to Gram-positive bacilli.

3.2. Molecular Identification of Bacterial Isolates

Out of four bacterial isolates, three isolated strains, i.e., QY4, QY5 PP784163, QY6, were identified as *Bacillus subtilis* NR_104873.1, *Bacillus subtilis* NR_104873.1 and *Bacillus subtilis* CP020102.1; one strain, QB2, was identified as *Bacillus cereus*. The length of *Bacillus subtilis* QY5 PP784163 NR_104873.1 was measured as 1538 base pairs.

The phylogenetic tree of *Bacillus subtilis* QY5 PP784163 (NR_104873.1), as depicted in Figure 4, was constructed using a neighbor-joining method that described the similarity index of the bacterial isolate to other species. The isolated bacterium showed 72% homology to the *Bacillus subtilis* strain ST15 (MK511833.1) and *Bacillus subtilis* strain ZB (KX450400.1).

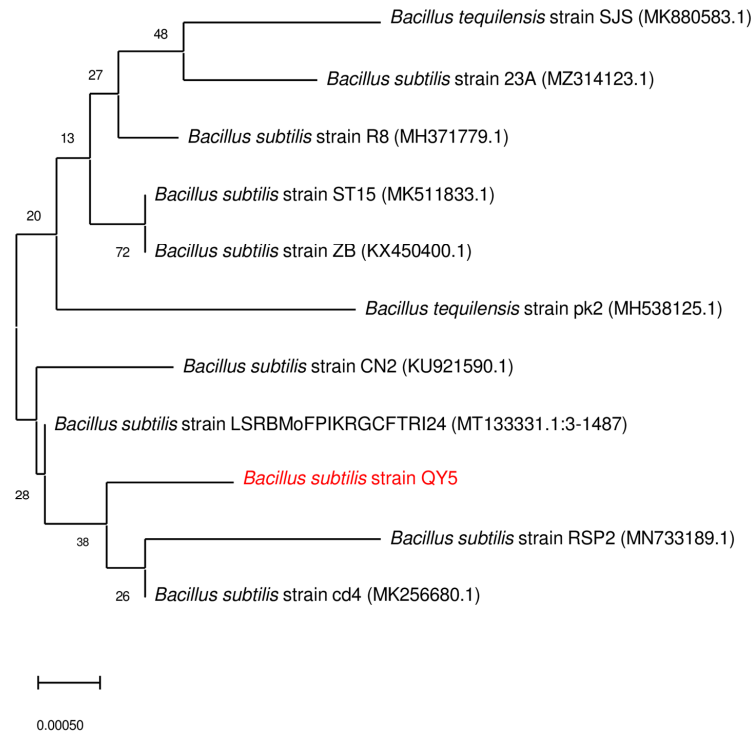


Figure 4. Phylogenetic tree of newly isolated bacterial isolate; *Bacillus subtilis* QY5 PP784163 NR_104873.1.

3.3. Ethanol and Glucose Tolerating Potential of Yeast and Bacterial Strain

Out of the four different concentrations of ethanol, *S. cerevisiae* reported growth at 10%. Beyond this concentration, no growth of yeast strain was observed. In contrast, for glucose concentrations, yeast growth was observed across all concentrations, i.e., 5–20%. Bacterial growth was observed at 5% ethanol concentration, with minimal growth observed at up to 10% ethanol. For glucose concentrations, growth was observed up to 20%, with the highest growth reported at 5%, as shown in Figure 5. A research report has recorded a genetically engineered strain of *S. cerevisiae* CAT-1 that can tolerate ethanol concentrations up to 14% [60]. At high concentrations of glucose and ethanol, yeast growth was reported to be negative. Thermo-ethanol tolerant bacterial strains isolated from fermented mango alcohol have reported ethanol tolerance up to 25% v/v in work by Kourouma et al. [61].

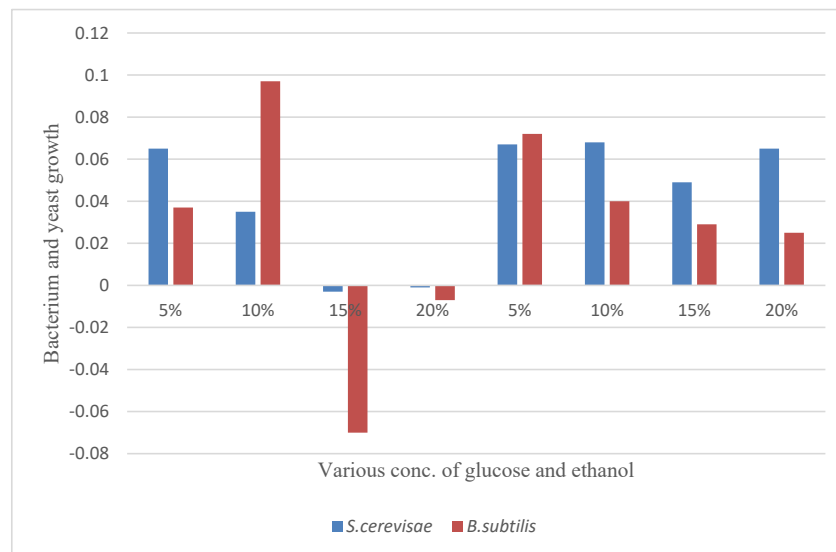


Figure 5. Glucose and ethanol tolerating potential of *S. cerevisiae* and *B. subtilis*.

3.4. Optimization of Physical Parameters for Ethanol Production in Submerged Fermentation

Both commercial and indigenous media were used to optimize ethanol production using the one-factor-at-a-time (OFAT) approach. Commercially available media YPD reported high titer of ethanol. Using commercial media, the effect of four parameters, temperature, pH, inoculum size and incubation time, was checked for their impact on ethanol yield. Preliminary experiments were carried out to find the values that gave good results. Afterward, the experiment of selecting the optimum temperature value was carried out. The optimal ethanol titer was observed at 35 °C, pH 4, with an inoculum size of 4%, after 6 days of submerged fermentation, as shown in Figure 6A. In contrast, PPW with indigenously produced amylase and cellulase produced the maximum ethanol at 37 °C, pH 5.5, with 4% inoculum size after six days of submerged fermentation (Figure 6B). Several studies have investigated the potential of organic waste, i.e., PPW, to produce ethanol using extremophiles and pH-tolerant microbes. They have optimized various physical and chemical parameters of the fermentation process to enhance its titer [62–65]. It is interesting to note that different fruit peels as lucrative substrate have contributed to good ethanol yield of 5.98 g/L, 10.1 g/L and 14.4 g/L after various time periods of fermentation using different microbial agents [66–68].

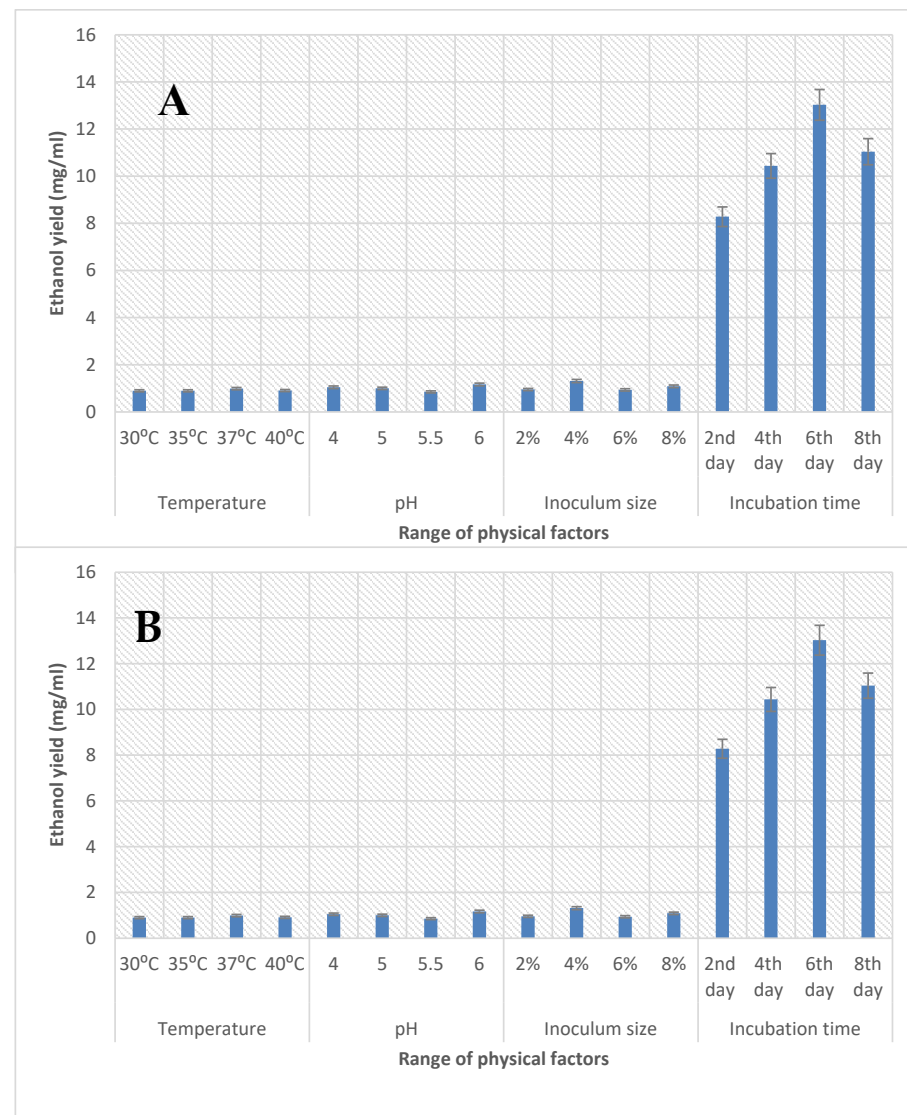


Figure 6. (A) Optimization of various physical factors to optimize ethanol yield using YPD media and (B) PPW media.

3.5. Compatibility of Bacterium and Yeast

Before initiating different routes to optimize ethanol production, the compatibility of both microbial strains was checked visually on Petri plates. Visible bacterial growth was observed around the sides of filter paper discs soaked with the culture fluids of the yeast, confirming that both microbial species can grow together, as shown in Figure 7. After confirming the compatibility of both strains, their potential to hydrolyze PPW for ethanol production was checked.

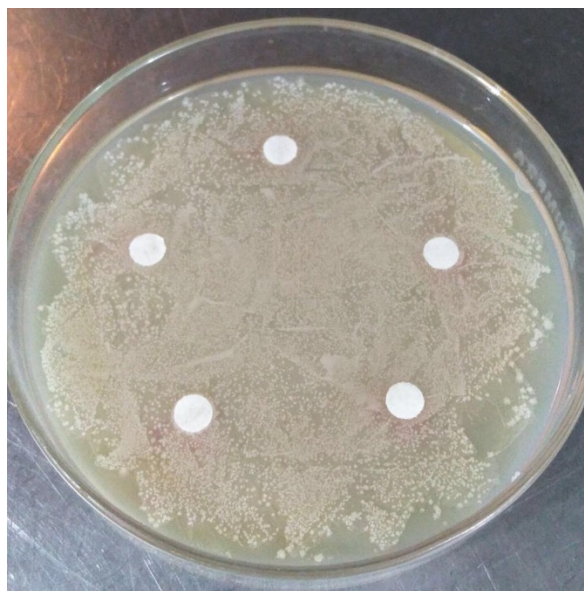


Figure 7. Compatibility of *B. subtilis* QY5 PP784163 (lawn) around the edges of filter paper discs inoculated with yeast culture.

3.6. Ethanol Quantification by HPLC

3.6.1. Consolidated Fermentation (Route A)

Consolidated fermentation is a promising strategy for bioethanol production because it combines three processes, i.e., enzyme production, biomass breakdown and fermentation, in one route. This strategy can decrease the cost of each separate process, which is one of the main hurdles in low-cost biofuel production. Hence, consolidated bioprocessing (CBP) generally utilizes a single microbe or consortium for enzyme production, hydrolysis and fermentation [69–73]. In the present study, a high ethanol titer was observed for consolidated fermentation. Both bacterial and yeast cultures were inoculated to hydrolyze the pretreated substrate, and they produced 0.4% ethanol content, as shown in Table 1. A recently published study utilized genetically engineered *B. subtilis* and *S. cerevisiae* enzymes in CBP using inexpensive plant biomass. They reported 21.5 g/L ethanol titer after four days of fermentation [74]. Another study also reported 21.7 g/L ethanol using optimized media of PPW through CBP [75]. Several research reports have reported efficient ethanol production via this effective approach CPB using cheap different plant biomass as a carbon source [76–79].

Table 1. Ethanol content of various experiments.

Sr #	Routes	Ethanol (%)
1	A	0.39
2		0.41
3		0.33

Table 1. Cont.

Sr #	Routes	Ethanol (%)
4		0.49
5	B	0.50
6		0.49
7		0.46
8	C	0.43
9		0.45
10		0
11	D	0
12		0
13	Standard	100

3.6.2. Separate Saccharification and Ethanol Fermentation (Route B)

Separate hydrolysis and fermentation (SHF) is an effective strategy to optimize the conditions of both enzymatic breakdown and hydrolysis, as both steps are spatially separated. The basic principle involved in this strategy is the separation of both reaction processes in separate reactors. For example, both enzymatic hydrolysis and fermentation occur at different optimal temperatures as enzymes. During SHF, enzymatic hydrolysis of biomass occurs first at the optimal temperature of enzymes, which is mostly 40–50 °C, whereas fermenting microbes cannot perform fermentation at this temperature; they are added at 30–40 °C to ferment the saccharified substrate at its optimal conditions [80–84]. In the present investigation, the first substrate was hydrolyzed by indigenously produced bacterial enzymes at 40 °C and then allowed to ferment by *S. cerevisiae* at 37 °C, which produced an ethanol yield of 0.50%. A research report by [85] has reported an ethanol yield of 33 g/L with SHF as a promising approach using cheap biowaste sugarcane. Another investigation has obtained 86% ethanol yield via SHF utilizing wheat straw as cheap carbon feedstock [86]. Several other reports have also reported high ethanol titer through SHF utilizing various plant biomass as cheap substrates [87–89].

3.6.3. Two-Step Saccharification Plus Ethanol Fermentation (Route C)

In this method, saccharification of the substrate was achieved in two steps, and the degraded substrate was then allowed to ferment by fermenting yeast. This route yielded an ethanol titer of 0.46%. In SSF, enzymatic breakdown and fermentation occurred in the same vessel. It has been reported that SSF can overcome many drawbacks of SHF as it requires less apparatus, less fermentation time and reduces the contamination chances [90–93].

The research report using simultaneous saccharification and fermentation (SSF) recorded 22.54 g/L of ethanol yield using PPW as a substrate. Another research report has observed a high ethanol yield of 116.5 g/L from PPW using SSF after 3 days of fermentation. Another study by [94] measured maximum ethanol production of 35 g/L using sweet potato by SSF under self-designed optimum conditions. Many research reports have recorded high production of second-generation bioethanol using SSF and different cheaper plant-based feedstocks [95–98].

3.6.4. Bacterial Saccharification and Fermentation (Route D)

In this route, only bacterial strain was used for both saccharification and fermentation, and no ethanol production was examined by HPLC. Few research reports have reported the ethanol yield by *B. subtilis* co-culturing with other fermenting microbes. The investigation by [99] has recorded a high biofuel yield of 1.1 g/L by using engineered strains of *B. subtilis* and *E. coli* and plant hydrolysate biomass. The report by [74] also examined the ability of engineered *B. subtilis* using CBP for the production of ethanol, up to 21.5 g/L from

inexpensive plant wastes. The research by [100] described the potential of co-culture of *B. subtilis* and *S. cerevisiae* for ethanol production of 5.8 g/L under optimal conditions. Few research reports have recorded the potential of engineered strains of *B. subtilis* or co-culturing with other fermenting microbes for biofuel production utilizing cheaper plant biomass [101,102].

Hence, a comparative analysis of various aforementioned routes provides very economical and feasible ways to produce bioethanol. Using indigenously produced enzymes could be a promising strategy to reduce the production cost of ethanol. Consolidated bioprocessing/Route A reported maximum ethanol yield and proved to be an effective route of all routes to enhance ethanol production. Other routes also reported cost-effective conditions for ethanol optimization and established the reliability of various conditions that can be beneficial for bioethanol production both at pilot and commercial scales. Recent advances in CBP have overcome many bottlenecks for second-generation ethanol production and reached up to 1.86 g/L/h of ethanol after the conversion of both solid and liquid fractions of sugarcane [103]. Another research report has recorded ethanol concentrations of 5.48 g/L utilizing optimum concentrations of banana peel as substrate [104].

3.7. Potential of Fermented Residual Cake by Proximate Analysis

To check the nutritional significance of residual fermentation cake as an animal feed supplement, this cake was examined for various useful compounds. The content of moisture, ash, fiber, crude protein, fat and carbohydrates was determined to appreciate its potential in animal feed supplements. The present study reports the application of submerged fermentation (SmF) as a bioprocess, where the nutritional prominence of PPW was upgraded for use as an animal feed supplement. The investigated SmF process was initiated by *S. cerevisiae* and *B. subtilis* species, which comprises a natural source of single-celled protein (SCP), fiber, various vitamins and minerals. Herein, the SmF of PPW reported a considerable rise in protein content by 17.45 and 18.24% compared to the control sample, which is 1.26%, as shown in Table 2. A massive increase in protein content due to the addition of yeast compared to control signifies the utilization of fermented residue as an animal feed supplement. A study has performed work on protein enrichment of PPW using *S. cerevisiae* via SSF and recorded a 12.5 to 21.8% increase in crude protein content. This study has proved the potential of fermented peels as a good source of cheap protein-enriched feed for livestock [105]. Another study has also reported an increase in the crude protein content of up to 13.62% of the fermented PPW from the addition of *S. cerevisiae* and *A. niger*, and therefore, fermented PPW could be used as a good source of cheap protein-enriched feed [106].

Table 2. Proximate analysis of substrate before and after inoculation of microbial species.

Sr #	Parameters (%)	Control (without any bacterial and yeast culture)	A	B	C	D
1.	Moisture	9.051 ± 0.12	7.290 ± 0.03	7.080 ± 0.05	6.771 ± 0.06	7.951 ± 0.11
2.	Ash	14.881 ± 0.07	7.600 ± 0.13	7.070 ± 0.03	7.231 ± 0.08	7.850 ± 0.12
3.	Crude fat	0.030 ± 0.12	2.881 ± 0.11	2.481 ± 0.04	1.930 ± 0.03	1.721 ± 0.17
4.	Crude protein	1.260 ± 0.23	17.450 ± 0.21	1.191 ± 0.11	18.241 ± 0.26	15.941 ± 0.15
5.	Crude fiber	0.210 ± 0.15	4.331 ± 0.16	3.590 ± 0.06	4.980 ± 0.17	4.750 ± 0.17
6.	Carbohydrates	74.781 ± 0.19	60.450 ± 0.11	82.180 ± 0.06	60.851 ± 0.15	61.791 ± 0.09

Values are presented as an average of triplicate values ± S.D.

Our study has recorded high values of carbohydrate content, i.e., 82.18 and 61.79%; therefore, PPW can be used as an attractive feedstock and cheap animal feed supplement. The research report by [107] has recorded 84.64% of carbohydrate content from PPW, while another study reported 65.47% of total carbohydrates on a dry weight basis via proximate analysis [108]. After bacterial- and yeast-submerged fermentation using PPW

as substrate, lipid content was also found satisfactory. After submerged fermentation, the rise in fat content was found to be 2.88 and 2.48%, while in the control, it was observed as 0.03%. A study by [109] has investigated the maximum lipid content in rice bran via fungal-based solid-state fermentation. The results of this study show that PPW can be nutritionally upgraded with the help of *S. cerevisiae* for animal feed. After the fermentation, the nutritional value of fiber was also highly upgraded, i.e., 0.21% to 4.98% and 4.7%, which can be very effective in improving the digestibility and gut health of animals. A research report using brewer's spent grain as substrate has reported improved fiber and protein content and recommended its use as proteinaceous animal feed [110].

4. Conclusions

Conclusively, the high quantity of carbohydrates, particularly starch, can serve as an attractive feedstock for different bioprocesses for sustainable production of biofuels. The universal trend for the production of valuable goods and sustainable energy needs to alter PPW from waste into a valuable resource. The current study utilized the PPW for bioethanol production with concomitant production of animal feed supplements while presenting the solution of waste management for the food industry. The proposed work plan had been performed with one feedstock using local microbial species. Advanced work on blending diverse feedstock while employing robust microbial agents could have a significant impact on the yield of value-added compounds in economical and practical ways.

Author Contributions: Conceptualization, Q.M., U.I. and P.M.; Methodology, Q.M., U.I. and P.M.; Software, U.I. and P.M.; Validation, U.I., N.J., A.S. and P.M.; Formal analysis, Q.M., U.I., N.J. and P.M.; Investigation, Q.M., U.I. and P.M.; Resources, A.S. and P.M.; Data curation, N.J. and P.M.; Writing—original draft, U.I.; Writing—review & editing, Q.M. and P.M.; Visualization, Q.M., U.I., A.S. and P.M.; Supervision, Q.M. and P.M.; Project administration, Q.M., U.I. and P.M.; Funding acquisition, U.I. and P.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Sequencing data from this study were deposited at the NCBI Gene Bank under accession number PP784163, and the strains were deposited in the Microbial Stock at the Microbial Biotechnology Laboratory located at the Institute of Zoology, University of Punjab, Lahore, Pakistan, and Microbial Stock Conservation at the Department of Research and Development at Paktex Industries, Kamoke, Gujranwala, Pakistan, under conservation numbers MBLZPU-9011 and R&D/PI203-1, respectively. [NCBI] [<https://submit.ncbi.nlm.nih.gov/subs/?search=SUB14441665>] [PP784163], accessed on 27 August 2024.

Conflicts of Interest: The authors declare no conflicts of interest.

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