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Study Of Microbiota from Sweet Spices for Gluten Degradation and Probiotic Potential

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Abstract:

Gluten, a ubiquitous protein found in wheat and other cereals, is known to trigger celiac disease, wheat allergy, and type 1 diabetes in genetically predisposed individuals. The incomplete degradation of gluten by human digestive enzymes results in the accumulation of toxic peptides that elicit inflammatory and immune responses in the intestine. Therefore, microbial enzymes capable of degrading gluten are of great therapeutic importance for gluten-sensitive individuals. In this study, bacterial isolates were obtained from sweet spices and evaluated for their gluten-degrading potential. A total of sixteen bacterial isolates were screened, among which three promising strains—identified as Bacillus cereus, Bacillus subtilis, and Pantoea cypripedii demonstrated significant gluten hydrolyzing ability. The purified enzyme, referred to as glutinase, efficiently degraded gluten into smaller, non-toxic peptides. Enzymatic assays confirmed the catalytic efficiency and stability of glutinase under simulated gastrointestinal conditions. Moreover, the selected bacterial isolates exhibited notable probiotic attributes, including tolerance to bile salts and low pH, as well as antioxidant activity, suggesting additional health benefits beyond gluten degradation. These findings highlight the potential of spice-derived microbiota as a source of novel gluten-degrading enzymes and probiotic candidates. The study opens new avenues for the development of safe and effective therapeutic supplements for individuals suffering from celiac disease and other gluten-related disorders.

Keywords: Gluten, Glutinase , Microbiota, Probiotic.

Introduction:

Gluten is a combination of grain storage proteins found in cereal grains. Wheat is the primary source of gluten in the human diet, having been domesticated around 10,000 years ago when hunter-gatherers settled in the Middle East.[1]Gluten is a fascinating combo of proteins that can be divided into two families based on their solubility in aqueous alcohols: gliadins and glutenins. Gluten proteins have a unique foundation amino acid structure and contain numerous glutamine (38%), proline (20%), and repeating PQpeptide sequences. Gluten, a grain protein, is incompletely digested by human proteolytic enzymes, resulting in immunogenic peptides that accumulate in the GI tract. [1]Gluten proteins play an important role in the food and baking sectors due to their viscoelastic properties. Some gluten proteins hydrolyze into immunogenic peptides that are poorly digested by gastrointestinal proteases such as pepsin, trypsin, and chymotrypsin, causing illness insensitive people.[3]Gluten plays a crusial role in making bread from wheat flour. Wheat contains around 50%prolamine, a type of alcohol-soluble protein having different places of origin. Alcohol-soluble fractions in wheat, barley, rye, and oats are known as gliadin, hordein, secalina, and avenin, respectively. When wheat flour is combined with water and physically homogenized for the creation

of bakery products, gluten is created when the gliadin proteins and glutenin flour hydrate and combine to form a protein complex through the use of disulfide, hydrogen, and Vander Waals bonds. [4]

Gluten Associated Disorders:

Three discrete disorders can be distinguished among wheat/gluten-related illnesses: autoimmune, allergic, and neither autoimmune nor allergic . The most common autoimmune gluten-related condition is coeliac disease (CD). It is a small intestinal disorder triggered by gluten and gluten-related proteins and impacted by genetic and environmental factors . Wheat allergy (WA) is characterised by an IgE and non-IgE intermediated immune response that causes an allergic reaction in certain people when they come into contact with, inhale, or consume foods containing wheat but not essentially other grains like barley or rye. However, certain individuals may have IgE-cross reactivity to other cereals . [10]

Methods :

Collection of sample:

Collecting a different type of spices, including sesame seed, flaxseed, fennel seed, cardamom, & black pepper. Use sterile sampling tools, such as sterile gloves or forceps, to further process.

Sample processing:

The collected samples underwent a thorough preparation process. Initially they were rinsed with sterile distilled water to remove any surface contaminants. The outer layer was then aseptically remove and the innermost Portion was excised using a Sterile knife. The extracted, tissue was subsequently homogenized in sterile saline solution using a sterile glass rod , ensuring aseptic condition throughout the Process.

Enrichment of sample:

Inoculate the processed sample into sterile Minimal Broth supplemented with gluten. Incubate the inoculated broth at 37°C for 4-5 days.

Isolation and Selection of gluten degrading bacteria:

The enriched sample were streaked onto Sterile minimal agar plates supplemented with gluten and incubated at 37°c for 24-48 hrs. After incubation, the plates were examined for zone of hydrolysis (Clearance), which indicate the presence of gluten degrading bacteria.

Colony and Morphological characterization Isolates:

The isolated were characterized based on their colonial and morphological features, including colony shape, size, colour, texture as well as cellular shape, size, arrangement and staining properties.

Biochemical characterization of isolates:

Catalase test:

The Catalase test detects the presence of the enzyme catalase, which break down hydrogen Peroxide (H202) into water(H2O) and oxygen (O2) .The release of oxygen Produces a visible bubbles, indicating a positive Reaction.

Oxidase test:

The Oxidase test detects the presence of enzyme cytochrome c oxidase, which is involved in the electron transport chain. This enzyme catalyses the transfer of electron from a reduced substrate to oxygen. This test differentiates oxidase- positive and oxidase - negative bacteria.

Endospore Staining:

Endospore staining detects the presence of endospores in bacteria by using Malachite Green to stain the endospores green and Safranin to counterstain the vegetative cells pink.

Nitrate Reduction test:

Nitrate Reduction Test detects bacteria that produce nitrate reductase, an enzyme that converts

nitrate (NO3-) into nitrite (NO2-), nitrogen gas (N2), or ammonia (NH3).

Identification of the Isolate:

The identification of the isolate was accomplished by combining colony morphology, Gram staining characteristics, and biochemical characterization. The unique characteristics observed were then matched with known bacterial species using Bergey's Manual, a comprehensive reference guide for bacterial identification. By integrating these methods, the isolate was successfully identified.

Enzyme profile of isolate:

Gluten degrading enzyme extraction:

The method used can vary depending on the source of the enzyme and its intended use. Take a sterile gluten containing plate and streak the sample on it ,plates incubate at 370C for 24 hrs observe the zone of hydrolysis.

Then pick a colony that showing zone of hydrolysis and inoculate in 100 ml sterile minimal salt broth containing gluten . Incubation of minimal salt broth in rotary shaker for 3-4 days .After incubation, centrifuge the broth in cooling centrifuge at 10,000 rpm for 15 min .The supernatant (the liquid portion) is collected, as it contains the dissolved enzymes referred as crude enzyme .

Gluten degrading enzyme Purification:

The extracted enzyme, referred to as the crude enzyme, was subjected to purification using the Ammonium Sulphate method. A 20 ml sample of the crude enzyme was placed in a flask, and an equimolar amount of Ammonium Sulphate was added. The Ammonium Sulphate solution was prepared at a concentration of 15:25. The flask was then incubated overnight in a refrigerator at 4°C. The following day, the enzyme extract was centrifuged in a cooling centrifuge at 10,000 rpm for 15 minutes. The resulting precipitate settled at the bottom of the tube and was referred to as the purified enzyme.

Gluten degrading enzyme Estimation:

The Folin-Lowry method is a widely used technique for estimating protein concentration. It involves reacting proteins with copper ions and Folin-Ciocalteu reagent, producing a blue color that is proportional to protein concentration. The method has high sensitivity, detecting concentrations as low as 1-100 µg/mL, and works well for pure proteins, enzyme solutions, and biological samples.

Gluten degrading Enzyme Immobilization:

The purified enzyme was immobilized using the glutaraldehyde cross-linking method. Initially, a solution of glutaraldehyde (2.5% v/v) was prepared in 50 mM phosphate buffer, pH 7.0. Then, 1 g of sodium alginate was dissolved in 100 ml of distilled water and mixed with 10 ml of the purified enzyme solution. The mixture was stirred gently for 30 minutes to ensure uniform distribution of the enzyme. The glutaraldehyde solution was then added dropwise to the enzyme-alginate mixture while stirring. The mixture was allowed to cross-link for 2 hours at room temperature. The resulting immobilized enzyme beads were washed with distilled water and stored in 50 mM phosphate buffer, pH 7.0, at 4°C for further use.

Enzyme Degradation Percentage:

Enzyme percent degradation refers to the proportion of a substrate that has been broken down by an enzyme, expressed as a percentage. It quantifies the enzyme's efficiency in degrading a specific substrate over a given time. The extent of degradation is measured by comparing the amount of substrate before and after enzymatic action.

Probiotic potential of isolates:

Probiotic potential refers to the ability of microorganisms to confer health benefits when consumed. Probiotics maintain a healthy gut

microbiota balance, essential for digestion, immunity, and well-being. They exert benefits through competition with harmful bacteria, digestion support, gut barrier strengthening, and immune system modulation. Probiotic potential is assessed through tests evaluating survival, colonization, and health benefits, including acid tolerance, bile salt tolerance, and antioxidant potential.

Acid Tolerance:

pH optimization refers to the process of determining and maintaining the ideal pH level where a gluten-degrading enzyme exhibits the highest activity and stability. It important to check the Optimal pH for Maximum Activity, Maintain the Structural Integrity of the Enzyme, and Improve Enzyme-Substrate Interaction. Prepare enzyme reaction mixtures at a broad pH range (e.g., pH 3.0 to 10.0) using different buffer systems. Measure enzyme activity using a spectrophotometric assay at 280 nm. Identify the approximate pH range where enzyme activity is highest.

Bile Tolerance:

Bile salts tolerance test is used to assess the ability of microorganisms (especially probiotics and gut bacteria) to survive and grow in the presence of bile salts. Growth rate of bacterial cultures was determined in MRS broth containing different levels (0, 0.1, 0.3, 0.5 and 0.7%) of bile salts (oxgall). Freshly prepared cultures were inoculated (1%) into medium and incubated at 37°C for 24 h under anaerobic condition, except for Streptococcus thermophilus, which was incubated under aerobic condition. Optical densities were measured spectro-photometrically at 620 nm after 0, 3, 5 and 24 h.[22]

Zymography:

To test the presence of protease activity by zymography , the sample was mixed with non-reducing SDS PAGE sample buffer (40 mMTrisHCl pH 6.8, 1% SDS, 2% glycerol and 0.01% bromophenolblue) and applied without boiling to a 10% polyacrylamide gel containing 0.1% SDS and 1% gluten. After electrophoresis, the gel was washed in 2.5% Triton X-100 solution for 30 min at room temperature followed by incubation in a reaction buffer containing 50 mMTrisHCl pH7.5, 1% Triton X-100 and 25 mM CaCl2 at 37C for 18 h. The gels were developed using Coomassie Brilliant Blue R solution and protease activity appeared as white band in blue background.[21]

Result:

Isolation of gluten degrading bacteria:

Total 12 isolates are isolated from 5 spices sample. Out of 12 isolates 2 isolates are isolated from *fennelseed*, 4 isolates are isolated from *flexseed*, 4 isolates are isolated from *sesameseed* , 1 isolates are isolated from *cardimum* and 1 isolates are isolated from *blackpaper*.

Zone of gluten specific media:

From 12 isolates five isolates showing zone of clearance that means ability to degrade the gluten.

Figure: Isolate showing zone of hydrolysis

Biochemical Characterization of isolate:

Sr.no	Isolated code ⇨	Fe/mini/2	Fl/mini/1	Se/mini/3	Ca/mini/1	Bp/mini/1
	Biochemical Test ⇩					
1.	Gram	Gram positive	Gram positive	Gram positive	Gram positive	Gram positive
2.	Motility	Motile	Motile	Motile	Motile	Motile
3.	Indole Test	Negative	Negative	Negative	Negative	Negative
4.	MR Test	Negative	Negative	Positive	Negative	Positive
5.	VP Test	Positive	Positive	Positive	Positive	Positive
6.	Citrate Test	Positive	Positive	Negative	Positive	Negative
7.	Gelatinase Test	Positive	Positive	Positive	Positive	Positive
8.	Oxidase Test	Positive	Negative	Negative	Variable	Negative
	Maltose	Positive	Positive	Positive	Positive	Positive
1	Manitol	Positive	Positive	Negative	Positive	Negative
11.	Casein hydrolysis	Positive	Positive	Positive	Positive	Positive
12.	Catalase Test	Positive	Positive	Positive	Positive	Positive
13.	OF Test	Positive	Positive	Positive	Positive	Positive
14.	Starch hydrolysis	Positive	Positive	Positive	Positive	Positive

Identification of isolates:

According to gram staining ,colony morphology ,and biochemical characterization and classification using bergey's manual the test isolate **Fe/mini/2** may be identified as a ***Bacillussubtilis***,**Se/mini/3** may be identified as *a* ***Bacillus cereus*** **.**The bacterial isolate FL_MINI_1 was identified as ***Pantoeacypripedii*** based on 16S rRNA gene sequencing showing 97.49% sequence similarity with reference sequences in the NCBI GenBank database**.**

Enzyme profile of isolates : Out of 12 isolates 5 isolates where selected for enzyme extraction. The isolates were selected on the basis of ***protease*** test, which showing the zone of surrounding the colony. [23]

Gluten degrading enzyme extraction: For the extraction of gluten-degrading enzymes, isolates exhibiting a clear zone surrounding the colony were selected. A suspension of each selected isolate was prepared and inoculated into minimal broth containing gluten as the sole nitrogen source. The cultures were then incubated at 37°C for 24 hours.

Gluten degrading enzyme purification: The ammonium sulphate precipitation method where performed for the purification of gluten degrading enzyme.

Glutenase Enzyme:

Sr.no	Sample name	Total protein concentration	Enzyme activity	Degradation percentage
1	Fennel seed	0.09218 mg/ ml	0.016	-
2	Flax seed	0.08978 mg/ ml	0.015	76.75%
3	Sesame seed	0.08107 mg/ ml	0.014	76%

Probiotic potential of isolate:

Acid tolerance:

Enzymes are affected by changes in pH. The most favorable pH value – the point where the enzyme is most active – is known as the optimum pH. This is graphically illustrated.The effect of pH on enzyme activity was analyzed by measuring the reaction rate at different pH levels.The highest enzyme activity (~2.163) occurs at pH 2, indicating this may be the optimal pH. Activity decreases at pH 4 but rises again at pH 5 and 8, suggesting some stability in this range.Activity drops at pH 10, indicating reduced efficiency at higher pH levels.The enzyme functions best in acidic conditions (pH 2-5) but loses activity in highly basic environments.[24]

Bile salts tolerance:

The bile salt tolerance of the Bacillus strain was evaluated by measuring optical density (OD600) after 24 hours of incubation in different concentrations of bile salts (1%–4%). The results indicate variations in growth across different concentrations, reflecting the strain's ability to survive under intestinal conditions.Growth in Control (0% Bile Salts) "In the absence of bile salts, the *Bacillus* exhibited a normal growth pattern with an OD600 value of 3.1035 after 24 hours, serving as the baseline for comparison.Effect of Bile Salts on Growth Upon exposure to bile salts, variations in growth were observed. At lower concentrations (1%–2%), the strain maintained relatively high OD600 values, indicating good tolerance. However, at higher concentrations (3%–4%), a gradual decline in growth was noted, with OD600 decreasing from(-0.1132) to(-0.1019) At (1% - 2 %) bile salts, a significant reduction in growth was observed, with an OD600 of 0.2519 & 0.2022 suggesting good inhibition."

Zymography: Zymography is a technique used to visualize and analyze the activity of proteolytic enzymes (proteases). It combines SDS-PAGE (a method for separating proteins based on size) with a substrate that the enzyme can degrade. The clear band were observed in zymography gel after incubation, indicate presence of active Proteases that capable to degrade gluten . The intensity of clear band suggest multiple enzyme with varying activity .clear bands confirm indicate areas where a protease has digested its substrate, allowing for the identification and quantification of proteolytic activity.

Conclusion:

This study isolated gluten-degrading bacteria from sweet spices (fennel, flaxseed). Twelve strains were obtained, with Bacillus spp. (B. licheniformis, B. subtilis, B. cereus) showing strong gluten hydrolysis. Enzyme activity was confirmed via casein agar (protease zones) and quantified using the Folin–Lowry method. Three isolates demonstrated significant gluten degradation. These bacteria, naturally present in spices, may transfer to the human gut and aid gluten digestion. Spices also provide fiber, supporting gut health and beneficial microbiota.

Future work could focus on enzyme purification for direct application, such as coating wheat grains to reduce gluten content, offering potential treatment for gluten-related disorders.

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