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Original Research Article

Isolation of Gluten Hydrolyzing Probiotic Bacteria

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ABSTRACT

Gluten, a common component in the human diet, is capable of triggering coeliac disease pathogenesis in genetically predisposed individuals.Coeliac disease is characterized by intestinal inflammation caused by gluten. Mammalian digestive enzymes are only partly capable of cleaving gluten producing residual fragments which are capable of inducing toxic responses in patients with coeliac diseases.It is a common fact that fermented food like curd is a rich source of probiotics(good bacteria) that are beneficial for the intestinal functioning. The current project was undertaken to isolate gluten hydrolyzing probiotic bacteria from functional food like curd. Gluten used was extracted from wheat dough. Bacterial isolates obtained from curd were cultured and maintained on MRSA agar. Out of the 8 isolates, growth of 6 was supported by 0.5% gluten solution (acting as Carbon as well as Nitrogen source). Thus, the gluten hydrolyzing probiotic strains were further subjected to biochemical tests for identification. The isolates in isolation or combination could be used in treatment of patients suffering from coeliac diseases (CD).

INTRODUCTION

Wheat contains a complex mixture of proteins that have unique ability to form viscoelastic dough when flour is mixed with water. [1] Gluten, the protein component of flour which gives the dough elasticity and strength, can be defined as the rubbery mass which remains when wheat dough is washed to remove starch granules and water soluble constituents. [2] Gluten is a general name for the proteins found in wheat (wheat berries, durum, emmer, semolina, spelt, farina, farro, graham), rye, barley and triticale. Gluten helps foods maintain their shape, acting as glue that holds food together and can be found in many types of foods, even ones that would not be expected. For e.g. oats as cross-contact may occur when oats are grown side-by-side with wheat, barley or rye. Pastas, Noodles (Note: rice noodles and mung bean noodles are gluten free), Breads and Pastries, Cereal and Granola, Breakfast Foods, Sauces & Gravies (many use wheat flour as a thickener), etc. [3-5] When people with celiac disease(s) eat, their body mounts an immune response that attacks the small intestine damaging the villi, thus lowering the nutrient absorption. Celiac disease can develop at any age after people start eating foods or medicines that contain gluten. Left untreated, celiac disease can lead to additional serious health problems. These include the development of other autoimmune disorders like Type I diabetes and multiple sclerosis (MS), dermatitis herpetiformis (an itchy skin rash), anemia, osteoporosis, infertility and miscarriage, neurological conditions like epilepsy and migraines, short stature, and intestinal cancers. Currently, the only treatment for celiac disease is lifelong adherence to a strict gluten-free diet. People living gluten-free must avoid foods with wheat, rye and barley, such as bread and beer. Ingesting small amounts of gluten, like crumbs from a cutting board or toaster, can trigger small intestine damage. [3-5]

Laboratory studies suggest that probiotics may contribute to gastrointestinal health specifically in patients with celiac disease by fortifying the protective mucus layer that lines the gastrointestinal tract, dampening the inflammatory response caused by gluten ingestion, decreasing intestinal permeability (the leakiness of the intestinal surface) in response to gluten, and possibly by aiding in gluten digestion. [4,6-9]

For commercial gluten production, the gluten is dried with high temperature air, whereas in the laboratory, freezing and vacuum drying have been reported to produce gluten with better functionality for bread making. [5, 10-16] Despite the ubiquity of wheat in the diet and the importance of gluten proteins for patients with CD, the bacteria that are involved in gluten metabolism and the role of probiotics in this metabolic process remain unknown. Accordingly, the aim of this study was to isolate lactic acid bacetria from curd and screen strains which can metabolize of gluten proteins.

MATERIALS AND METHODS

1. Isolation of probiotic strains from functional food

One gram of curd sample was added to 10 mL of saline, is 10:1 dilution. One ml sample from 10:1into another test tube having 9 mL of saline is10:2dilution.Similarly,dilutions to the power-7were carried out. 100 microliters of each dilution was spread plated on Nutrient agar and incubated at 37°C for 24 hrs. The CFU/ml was enumerated.

2. Gluten Extraction

To 100 gm wheat flour, approximately 30 mL of water was added to ready the dough, which was then soaked in water for 40 minutes to remove excess starch. The dough was also washed under running tap water to remove all of the starch present in it. A visco-elastic mass was obtained which is the gluten protein. To obtain free flowing powder of gluten, the visco-eleastic mass was cut into fine pieces (about 1cm x 1cm) and oven dried at 100°C till all the water content was evaporated i.e. it became crispy. The dry mass obtained was ground to obtain a fine powder, which was stored at room temperature in an air tight container for as long as it is required to avoid contact with moisture in the air.

3. Screening for gluten hydrolyzing probiotic isolates

The 8 cultures isolated from curd were inoculated into the following media and incubated at 37° C for 24 hours.

Media	Source of Carbon	Source of Nitrogen	Justification
1	NB	NB	To ensure all isolates are growing
2	Dextrose	Gluten	To replace the nitrogen source with gluten and check if probiotics can digest the same
3	Gluten	Gluten	To check if the strains are capable of digesting gluten with no other component acting as C or N source

*Media 1 is Nutrient Broth (NB). Media 2 is gluten-dextrose solution where 0.5% gluten in 1% dextrose solution is prepared. Media 3 is only gluten solution where 0.5% gluten solution was prepared.

4. Inoculation and Growth in 0.5% Gluten and Gluten-Dextrose Solution

About 0.025gm of gluten was added in 5mL of distilled water and in 5mL of 1% dextrose solution. Each of the 8 different cultures was inoculated into the gluten and gluten-dextrose solution. These test tubes were incubated at 37°C and observed over a period of time for growth in form of turbidity. Master slants of each strain were maintained at 4°C

5. Biochemical identification of isolated gluten hydrolyzing probiotic strains

For identification of bacterial isolates the following biochemical tests i.e. Methyl Red, Vogues-Proskauer, Catalase, TSI Agar (Triple Sugar Ion Agar) were performed [Table 1]

RESULTS AND DISCUSSION

Culture was isolated from fermented milk since it is known to have good digestive properties. The presence of microbes in milk is what results in the

formation of curd. Table 2 indicates the CFUs obtained on serial dilution of curd.

As expected number of colonies decreased with increase in dilution factor, colonies with distinct colony morphologies were identified and transferred on NA plates using sterile toothpicks. The colony characteristics were studied as enlisted in Table 3.

Once the isolates were collated and cultures, they needed to be screened for gluten metabolism. From 100gms wheat flour 30gms of gluten in visco-elastic mass was extracted. Thus the wet weight content of gluten is 30% in wheat flour. Post drying the 30gm was reduced to only 10gm, thus dry weight content of gluten in wheat flour is 10%.

The isolates were cultured in 2 sets of medis (as displayed in Table 4).To 5ml of sterile media in 20ml test tubes, 0.1ml of isolates (with A 540 nm = 0.1) was added and incubated for 24-48hours. All isolates indicated growth in the form of turbidity except GNKC/IC/63 and GNKC/IC/65 for Solutions A and B respectively.

Literature sites usage of gluten agar to isolate gluten hydrolyzing microorganisms, but our broth method is relatively faster and easier too. To learn the metabolic characteristics of the gluten hydrolyzing strains, all the isolates were subjected to some biochemical tests. The observations are reported in Table 5.

Comparing the results of the biochemical tests it can be deduced that observed colonies could be glucose and lactose and/or sucrose fermenting Enterobacteriaceae species or *Streptococcus* species. These kinds of projects have been performed before. Currently probiotics are being used in sour dough fermentation as a way to find foods good for CD patients.

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Table 1: Biochemical identification of isolated gluten hydrolyzing probiotic strains

Biochemical tests	**Observation(s)**	**Inference (probable strains)**
Methyl Red	Red (++)	*E. coli, Yersinia* sps, etc.
	Yellow (-)	*Enterobacter aerogenes, Klebsiella pneumoniae,* etc.
Vogues-Proskauer	Pink-red (++) Rust(+)	*Enterobacter aerogenes, Klebsiella pneumoniae,* etc.
	Copper (-)	*E. coli, Yersinia*, etc.
Catalase	Copious Bubbling (++)	*Enterobacteriaceae* (*Citrobacter*, *E. coli*, *Enterobacter*, *Klebsiella*, *Shigella*, *Yersinia*, *Proteus*, *Salmonella*, *Serratia*), *Pseudomonas*, etc.
	No or very few bubbles (-)	*Streptococcus* and *Enterococcus*
TSI Agar (Triple Sugar Ion Agar)	**(slant/butt)**	**Interpretation**
	Red/Yellow	Glucose fermentation only, peptone catabolized.
	Yellow/Yellow	Glucose and lactose and/or sucrose fermentation.
	Red/Red	No fermentation, Peptone catabolized.
	Yellow/Yellow with bubbles	Glucose and lactose and/or sucrose fermentation, Gas produced.
	Red/Yellow with bubbles	Glucose fermentation only, Gas produced.
	Red/Yellow with bubbles and black precipitate	Glucose fermentation only, Gas produced, H_2S produced.
	Yellow/Yellow with bubbles and black precipitate	Glucose and lactose and/or sucrose fermentation, Gas produced, H_2S produced.
	Red/Yellow with black precipitate	Glucose fermentation only, H_2S produced.
	Yellow/Yellow with black precipitate	Glucose and lactose and/or sucrose fermentation, H_2S produced.

Table 2: Isolation of Probiotic Strains from Functional Food

Probiotic Source	**Dilution**	**No. of colonies**
Curd	10^{-1}	>300
	10^{-2}	>300
	10^{-3}	90
	10^{-4}	12
	10^{-5}	4
	10^{-6}	1

Table 3: Colony Characteristics of Isolates

Characteristics	**GNKC/IC /51**	**GNKC/IC/ 52**	**GNKC/IC/ 54**	**GNKC/IC/5 5**	**GNKC/IC/ 62**	**GNKC/IC/ 65**
Size (mm)	Point	Point	Point	>3mm	Point	3mm
Chromogenesis	Creamish	Creamish	Yellow	White	Yellow	White
Margin	Entire	Entire	Entire	Undulate	Entire	Undulate
Elevation	Flat	Flat	Flat	Flat	Convex	Flat
Shape	Circular	Circular	Circular	Irregular	Circular	Irregular
Consistency	Mucoid	Mucoid	Mucoid	Mucoid	Mucoid	Mucoid
Opacity	Opaque	Opaque	Translucent	Translucent	Opaque	Opaque
Surface	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth

Table 4: Screening for growth of isolates supported by gluten

Isolate	Solution A (Distilled Water + Gluten)	Solution B (Dextrose Solution + Gluten)
GNKC/IC/51	+	+
GNKC/IC/52	+	+
GNKC/IC/54	+	++
GNKC/IC/55	+	+
GNKC/IC/62	+	+
GNKC/IC/63	-	+
GNKC/IC/64	++	+
GNKC/IC/65	+	-

+: indicates growth, ++ indicates higher growth

Table 5: Biochemical characterization of the gluten hydrolyzing isolates.

Tests	GNKC/IC/51	GNKC/IC/52	GNKC/IC/54	GNKC/IC/55	GNKC/IC/62	GNKC/IC/65
Methyl Red	-	-	No change	No change	+	No change
Voges-Proskauer	-	-	-	-	-	-
Catalase Test	++	++	-	-	++	-
TSI Agar Test	Glucose & lactose &/or sucrose fermentation.					

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