Analysis of enzymatic reduction of red lentil antinutrient trypsin inhibitors by *Lactobacilli* isolated from *Idli* batter

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Abstract **- Grains are a substantial source of macronutrients and energy for humans. In grains, red lentil is rich source of dietary protein but contains some anti-nutritional factors (ANFs), especially trypsin inhibitors (TIs) which hampers nutrient absorption and hence decrease nutritional value. Fermentation with lactic acid bacteria is the traditional and most popular way to improve the nutritional value of foods stuff. In present study, a strain of *Lactobacilli* was selectively screened and isolated from the *idli* batter a traditional fermented food of India and characterized for probiotic attributes like bile tolerance and antibiotic resistance. Depigmented and defatted red lentil powder was subjected to fermentation for 3 days and reduction of trypsin inhibitor content was analyzed quantitatively. Also, protein hydrolysis of lentil by proteases from *lactobacilli* was analysed on 10% SDS PAGE. Results revealed that isolate could be potential protease producing probiotic candidate hydrolyze the trypsin inhibitors both qualitatively and quantitatively and may be used for enhancing the nutritional value of lentil as starter culture as well as during food processing.**

Index Terms **- Fermented food, *Lactic acid bacteria*, Lentil, Trypsin inhibitors.**

I.INTRODUCTION

Legumes are gaining more interest for developing healthy and functional foods because of beneficial health use of micro and macro nutrients present in them. Red Lentil (Lens culinaris; Family: Fabaceae) is a yearly legume crop from Asia and other parts of the world. Furthermore, currently this species is widespread from Hindukush to Afghanistan and Ethiopia to Mediterranean countries (Faris *et al* 2012). It is well known for its lens-shaped edible seed, which has the main dietary part containing macro and micro-nutrients with bioactive molecules (FAO, 1988).

Lentils are a rich source of proteins. The major proteins in lentils are globulin (approximately 47% of the total seed proteins) and an adequate amount of albumin (Lombardi-Boccia et al.,2013). Sufficient composition of these proteins as well as essential amino acids in lentils offers an important and nutritious dietary source for low and middle-income nations (Hoover et al., 2010). Lentils also known to be a superior source of prebiotics (Dwivedi et al., 2014) and help to keep up the gut microbial environment by preventing gut-associated diseases (Fooks et al., 1999; Johnson et al., 2013). Lentils are relatively low in fat and sodium, but high in potassium content (Padovani et al., 2007) and because of that it is one of the best dietary foods for patients with obesity and cardiovascular diseases. Lentil seeds are an excellent vegetable source of iron. Earlier studies reported the intake of cooked lentil in the diet prevents iron deficiency in anaemia (Soltan, 2013). Overall, lentils are considered as one of the important dietary sources that has health-promoting effects on various illnesses. Apart from this, nutritional value of lentils was limited because of presence of anti-nutritional compounds (ANCs) such as phytic acid, protease inhibitors, tannins, and lectins present in them (Urbano et al., 2007; Nosworthy & House, 2017). In protease inhibitors (PIs) mainly like trypsin inhibitors (TIs) considerably reduces the protein digestibility if not properly removed or inactivated during processing (Boye et al., 2010).

Recent years, bacterial and probiotic peptidase widely used to reduce the antinutritional components from cereals and legumes (Francavilla et al., 2017;Wagh et al., 2018; Verni et al., 2020; Cristofori et al., 2020). Probiotics are live microorganisms which, when taken in adequate amounts beneficially affect the host by getting better intestinal microbial balance or Gastro intestinal flora (GIF) (Fuller 1989).It has been shown that fermentation carried out with probiotics increases nutritive value and digestibility of food (Nyanzi and Jooste, 2012; Rani et al., 2017; Petrova and Petrov, 2020). Also, probiotics as well as their consortia used to reduce ANCs from various food processes (Adeyemo and Onilude, 2013; Coda et al., 2015;Francavilla et al., 2017) as well as for improving the safety and shelf life of fermented food (Behera et al., 2018).

Traditional fermented foods are the best source of probiotics. However, in recent years, extensive focused research has been carried out on isolation of these probiotics especially LAB from traditional fermented foods on the basis of their sturdy and eco-friendly nature. These LAB's play an important role in numerous natural food fermentations such as cheese, curd, pickles as well as other traditional foods. Furthermore, they are very strongly associated with the human surroundings. This organism has also gained recognition as probiotics (Rivera-Espinoza and Gallardo-Navarro, 2010). In India, idli batter fermentation has been the focus of research studies since a long time. A large number of probiotic strains have been identified to be a part of the microflora responsible for fermentation of idli batter and they mainly include *L. coryneformis, L.lactis, Leuconostoc mesenteroides, L. fermentum, L.delbrueckii*, *Bacillus* sp.(Yajurvedi 1980, Sharma et al., 2020). Preparation methods, nutritive value and microbiology of idli have been broadly studied and well documented in previous studies (Venkatasubbaiah et al. 1984; Kanchana et al. 2008; Riat and Sadana 2009; Sridevi et al. 2010).

On the basis of above information, in this present study, we report a finding that *Lactobacilli* isolated from Idli batter having capability of significant hydrolysis of antinutritional trypsin inhibitors extracted from red lentils.

II.MATERIALS AND METHODS

Idli batter preparation and screening: For isolation of lactic acid bacteria (LAB) from idli batter, aseptically 1 gram of the batter was added to 9 ml saline solution (0.9 % w/ v Sodium chloride in distilled water) and blended thoroughly on an orbital shaker for 1 hour. An appropriate 10^{-7} serial dilution of the blended mixture was plated onto MRS - gelatin agar plate (pH 6.5) and incubated at 37°C for 48 h. This procedure was repeated at regular time intervals during the course of incubation. Well isolated gelatin diffusing colonies were picked up and suspended in10 ml MRS broth (pH 6.5) and incubated at 37°C for 48 h. After incubation the broths were twice centrifuged at 8000 rpm for 20 minutes. The supernatants were used as a source of crude protease. These isolated LAB's colonies were maintained on MRS agar slants. These supernatants were used for in plate screening of protease secreting bacteria.

For screening of proteases secreting isolates, gelatin agar plates were prepared as per previously reported method with some modifications (Majed et al., 2018).After, solidification of autoclaved gelatin agar, 5 mm wells were punctured and inoculated with 50 µl of centrifuged supernatant. After 48 hrs incubation, the gelatin agar plates were stained with Coomassie Brilliant Blue R 250 for 8 hrs and destained overnight using the destaining solution. The potent isolates were labelled as Isolate ID4 and ID7. These isolates were again screened by gelatin zymography.

Gelatin zymography:

Zymography is a simple and widely used technique which was employed for screening of protease activity. The electrophoresis was carried out according to the procedure of Laemmli (Laemmli, 1970). Briefly, in zymography, 1mm thickness separating gel was prepared by copolymerizing 0.1% gelatin in resolving gel solution (RGS) contained 375mM Tris/HCl (pH 8.8), 10% acryl/bis-acrylamide, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate (APS), and 0.04% (v/v) TEMED poured between the glass plates, then 50% butanol was added to remove the air bubbles and gel was allowed to polymerization. After 2 hr, the butanol was removed by repeated washing with distilled water. The stacking gel contained 4% acryl/bis-acrylamide, 126 mM Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 0.05% (w/v) APS, and 0.1% (v/v) TEMED was poured on top of the resolving gel and comb was inserted. After well formation, precast

polyacrylamide gel was placed in a Genei electrophoresis system Gel loading (sample) buffer containing 0.25 M Tris/HCl, 2% SDS, 10% glycerol, and 0.0025% bromophenol blue. The electrophoresis running buffer contained 24 mM Tris-Base, 192 mM Glycine and 0.1% (w/v) SDS (pH 8.3).

The gel loading dye mixed with 20 µl each Isolate ID4 and ID7 crude samples and gel was run at 70V for 40 minutes and 100V for 120 minutes. After running, the gel was carefully removed and renatured with 2.5% Triton X-100 (SRL, India) for 30 min. followed by rinsing with distilled water followed by incubation in activation buffer (50mM Tris- HCl, pH 7.8) at 37^0C for overnight. The gel was stained in 40% methanol/ 10% acetic acid/ 50% distilled water with 0.1% Coomassie brilliant blue R-250 for 4 hrs and destained in the same solution without dye.

The isolate ID4 showed good zymogram than ID7, hence was used for fermentation with Lentil powder.

Depigmentation and defatting of red lentils:

Dry mature seeds of red lentils (Masoor) were obtained from the local market in Aurangabad (MS) India. After dehulling, they were ground to a fine powder. Defatting and depigmentation were carried out thrice washes with hexane and acetone respectively according to our previous publication (Gadge et al., 2015). The recovered powder was air dried and used for further experimentation.

Fermentation of Lentil powder with isolate ID4:

The 2 gram defatted and depigmented lentil powder was weighed in triplicates into screw capped bottles. 5 ml sterile distilled water was added in it to become a paste. 1 ml each of the seed inoculum of Isolate ID4was added separately in two batches (ID-A and ID-B) and allowed to ferment for 3 days at 37^0C. Fermented digesta were taken aseptically for Trypsin inhibitor content determination as well as for in gel electrophoresis protein hydrolysis study after every 24 hrs. A control (ID-control) was set up in the rest of batch with addition of 1 ml of sterile distilled water in the powder instead of the inoculum.

Protein determination:

The protein estimation of all the samples was carried out according to the method of (Lowry et al., 1951) using Bovine serum albumin as standard.

In gel Electrophoresis analysis of Protein hydrolysis during fermentation:

For analyzing protein hydrolysis during fermentation with time, the proteins were extracted in ten volumes of 100 mM Tris- HCl buffer (pH 9) (Jarpa-Ferra et al., 2014) containing 1% PVP at 10°C overnight. The extracts were centrifuged twice at 8000 rpm for 20 min at 4 °C.

10 µg of proteins of Day 0, 1, 2 and 3 were resolved onto 10% SDS PAGE. Then electrophoresis was performed as discussed in above section.

Determination of trypsin inhibitors:

From the samples of 3 batches 0.01 g fermented digesta from each collected sample were weighed into a screw capped tube. In each tube 2 ml of 100mM phosphate buffer was added and kept on 200 rpm rotary shaker for 30 min. The suspension obtained was centrifuged at 8000 rpm for 20 min. The volume of each was adjusted to 5 ml with phosphate buffer. The test tubes were placed in a water bath, maintained at 37^0C. 6 ml of ice chilled 10 % Trichloroacetic Acid (TCA) solution was added to one of the tubes to serve as a blank. 2 ml of casein solution was added to all the tubes, which were previously kept at 37^0C. These were incubated for 20 min. The reaction was stopped after 20 min by adding 6 ml of TCA solution to the experimental tubes, after which centrifuged at 8000 rpm for 10 min. Absorbance of filtrate from sample and trypsin standard solutions was read at 380 nm on a spectrophotometer. The trypsin inhibitor in mg/g sample was calculated using the formula:

$$\text{Trypsin inhibitor mg/g} = \frac{\text{A STD-A sample x Dilution factor}}{19 \times \text{sample wt in g}} \times 1000$$

(AOAC, 1990).

Gram nature, morphology and Probiotic characterization:

The potent isolate ID4 was examined by Gram staining and morphology was evaluated microscopically. The bile salt tolerance activity of the isolate was determined by the direct plate method. Overnight culture of isolate ID4 was grown in MRS broth containing 0.5% Bile salt (HiMedia) incubated at 37^0C. The culture sample was taken and spread on MRS agar plate. The plate was incubated for 48 hrs at 37^0C.

To determine species specific breakpoint concentration of antibiotics as proposed by European

Food Safety Authority (EFSA), a strip comprised of a predefined quantitative gradient of specific antibiotic was used according to the manufacturer's instructions. Overnight Isolate ID4 culture was centrifuged at 6000 g for 20 min, washed twice, and re-suspended in phosphate buffer saline (PBS). Thus, prepared bacterial suspension (1 ml) was swabbed on MRS agar plate. Antibiotic disc from Himedia, India (IC006 Icosa universal-2) containing Amkacin (30mcg), Ampicillin (10 mcg), Amoxycillin (10 mcg), Cefadroxil (30 mcg), Cefoperazone (75 mcg), Ceftazidine (30 mcg), Ceftriaxone (30 mcg), Chloramphenicol (30 mcg), Ciprofloxacin (05 mcg), Cloxacillin (01 mcg), Co-trimoxazole (25 mcg), Erythromycin (15mcg), Gentamicin (10 mcg), Nadidixic acid (10 mcg), Netillin (30 mcg), Nitrofuranton (300 mcg), Norfloxacin (10 mcg), Pencillin (10 units), Tobramycin (10 mcg) and Vancomycin (30 mcg) were used for analysis.

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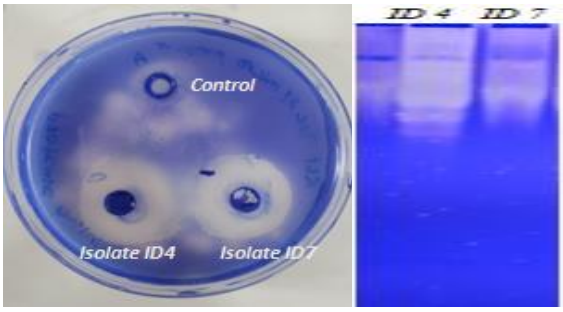
III.RESULTS AND DISCUSSION

Total 10 bacterial colonies showed dispersed gelatin precipitation on a MRS-Gelatin agar plate (data not shown). These dispersed colonies were repetitively subcultured and subjected to fermentation in the MRS medium at 37^0C, and after 48 hrs crude supernatant was used for qualitative determination of gelatin hydrolyzing analysis in the gelatin agar plate. After CBB staining gelatin hydrolysis was observed as a zone of clearance around the well. After repetitive experimentation, isolate ID4 and ID7 showing the most potent gelatin hydrolyzing enzyme production ability on a gelatin agar plate shown in Figure 1(A). This isolates again screened for gelatin zymography.

Gelatin zymography in qualitative analysis showed, Isolate ID4 having more protease producing ability than Isolate ID7 shown in Figure 1 (B). Hence Isolate ID4 is used for further experimentation.



(A) (B)

Fig.1. Screening of protease secreting isolates from Idli batter. In A- on gelatin agar Control- Distilled water, isolate ID4 and ID7 both shows zone of gelatin hydrolysis, In B- gelatin zymography of isolate ID4 and ID7.

After fermentation of Lentil powder with Isolate ID4, the extracted protein profile was analyzed on 10% SDS PAGE. Results revealed that from the first day, the isolate start protein hydrolysis as shown in figure 2. After 3 days it shows complete disappearance of protein bands.

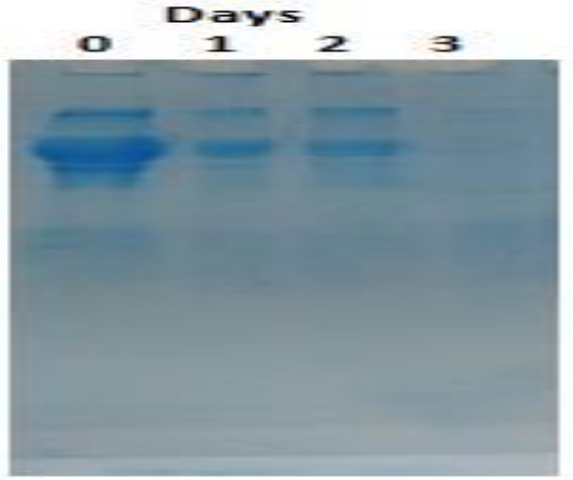


Fig. 2. 10% SDS PAGE protein hydrolysis analysis during fermentation of lentil powder with Isolate ID4. Lane 1- Extracted proteins at day 0, Lane 2- Extraction after 24 hrs, Lane 3-extraction after 48 hrs, Lane 3- Extraction after 72 hrs.

In earlier studies, Protease inhibitors found in lentils have been characterised as members of the Bowman-Birk family (Jarpa- parra, 2017). Trypsin inhibitors are proteins from bowman-birk family which binds and inactive digestive enzymes. In Dehulled lentil, trypsin inhibitor ranges between 1.17 to 4.25 mg/ gram (Pal et al., 2017). In current study, the trypsin inhibitors are found in between range. After fermentation till 3 days, trypsin inhibition content is considerably decreased as shown in table 1.

Table 1.Trypsin inhibitors (mg/g) content after fermented with Isolate ID4 inoculum

Sample	Day 0 (Control)	Day1st	Day2nd	Day3rd
ID-Control	2.34	2.26	2.11	2.04
ID-A	2.25	1.63	1.00	0.22
ID-B	2.28	1.59	0.97	0.29

Above table shows the reduction of trypsin inhibitors content after fermentation with *isolate ID4.*

Gram staining: The isolate ID4 that characteristic morphological cell categorized as Gram-positive bacteria. The Gram stain results revealed that the studied isolate from Idli batter was rod shaped *lactobacill.*

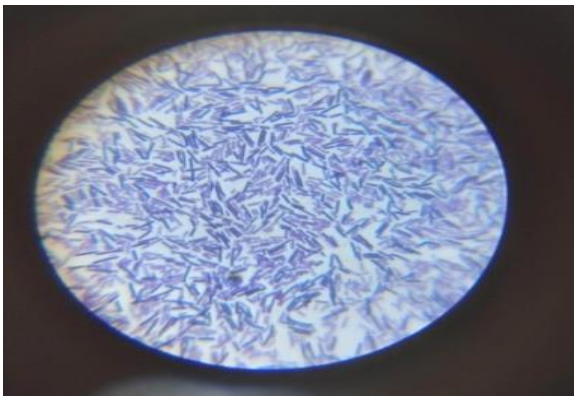


Fig.3. Gram nature of *Lactobacilli* isolated from Idli batter.

Bile tolerance: Bile tolerance is one of the essential criteria to be fulfilled for a LAB to be used as a probiotic which is its ability to resist the effect of bile salts in the gastrointestinal tract (GIT) (Lee and Salminen 1995). In current study, isolate ID4 was treated with 0.5% bile as it is the average concentration obtained in animal and human intestine during digestion process as reported in our previous study (Wagh et al., 2018). Studied *Lactobacilli* qualitatively showed good resistance against 0.5% bile salt after exposure at 48 hrs on plate as shown in figure 4.

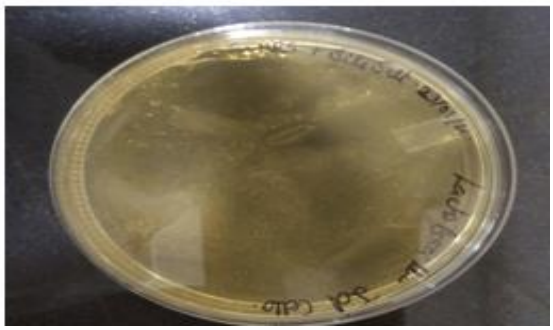


Fig.4. Bile salt tolerance

Antibiotic resistance or susceptibility of any strain is the essential criteria for evaluation of strains for their future application in food processing as a starter or adjuvant. The safety of strain is becoming prerequisite with antibiotic resistance as an emerging issue (Hummel et al. 2007). FAO/WHO guidelines have strongly recommended antibiotic susceptibility pattern of every probiotic strain prior to their consumption or applications in food (FAO/WHO 2002).

Studied *Lactobacilli* strain was screened for it's antibiotic sensitivity against discs obtained from Himedia. Studied strain showed variations towards the different antibiotics shown in Table 2.

Table 2.Antibiotic resistance profiles of *lactobacilli* against antibiotics

Antibiotics	Result	Antibiotics	Result
Amkacin	+	Co-Trimoxazole	-
Ampicillin	+	Erythromycin	+
Amoxycillin	++	Gentamicin	+
Cefadroxil	++	Nadidixic acid	++
Cefoperazone	-	Netillin	-
Ceftazidime	++	Nitrofurantoin	++
Ceftriaxone	+	Norfloxacin	+
Chloramphenicol	++	Penicillin	+
Ciprofloxacin	-	Tobramycin	-
Cloxacillin	-	Vancomycin	++

(Note-Zone of inhibition was calculated according to the table given by Clinical and Laboratory Standards Institute (CLSI, 2011).(++) Resistant, (+) Intermediate resistances, (-) Susceptible)

IV.CONCLUSION

Lactobacilli isolated from idli batter could tolerate bile salt and having antibiotic resistance properties. Based on these in vitro tests, there is high possibility that the strain would be used as probiotic. Studied strain significantly reduced the content of trypsin inhibitor during fermentation which can be useful in preparing a good lentil-based weaning food. However, this is very preliminary study; the isolated *Lactobacilli* need molecular identification, enzyme isolation and biochemical characterization as well as further investigation for using in vivo experiments to establish its potential health benefits.

V. CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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