Isolation, identification and characterization of culturable probiotic bacteria associated with fermented fish products

D.M.W.D.Divisekera[1*], H.R.W.M.G.C.Niyangoda[2]and S.S.K.Madage[1]

[1]*Industrial Technology Institute, Sri Lanka*

[2]*Uva Wellassa University, Sri Lanka*

Abstract

Probiotics are live microorganisms that have beneficial effects on the health of their host. Fermented fish is an important source of nutrients, which include essential proteins, unsaturated fatty acids, vitamins and minerals. The objective of this research is to isolate, characterize and identify viable probiotic bacteria in the fermented fish products. Serial dilutions were prepared from fermented samples, spread on Man Rogosa and Sharpe agar (MRS), and incubated at 37 ^{0}C for 48 hrs. Nine isolates were purified by streaking on MRS agar. All nine purified isolates were subjected to Gram staining, endospore staining and motility. Morphological characteristics of isolates were noted. Biochemical characterization was performed. Sugar fermentation patterns were studied. Tolerance of all isolates to different concentrations of salt (3%, 6%, 9%), temperatures (30 ^{0}C, 37 ^{0}C, 40 ^{0}C) and pH values (2, 3, 4) was studied. The molecular level characterization of isolates was carried out by extracting DNA and performing 16s rRNA sequencing. Among the nine isolates, seven were Gram positive and two were gram negative. All nine isolates were observed to be non spore forming and non motile and negative for Indole, Catalase, H_2S production and citrate utilization and could ferment fructose. All the isolates show optimum tolerance to 37 ^{0}C and 40 ^{0}C. The optimum tolerance pH was 3-4. At pH 2 the growth is weak. All of the isolates were showing the optimum growth at 6 % salt concentration. However, they can tolerate 3 % and 9 % salt. By molecular level identification, the isolates were identified as *Pediococcus acidilactici* (AB680157.1), *Lactococcus lactis* (CP006766.1), *Lactococcus lactis* (KJ690920.1) and *Weissella paramesenteroides* (HQ009793.1). Therefore, it provides strong evidence on survival of starter culture during the fermentation. Since, *Weissella paramesenteroides* was not in starter inoculums, it could be assumed that the starter culture was changed and improved during fermentation.

Keywords: Lactic acid bacteria, Probiotics, Fermented fish

**Corresponding author: mb.wasu@gmail.com*

Introduction

Fermentation is one of the oldest processing methods, which is used for the preservation of fish. According to Hwanhlem *et al* (2011), it extends the shelf life; enhances the unique taste, flavour and nutritional quality of the product. Fermented fish products are made by inoculating probiotic Lactic Acid Bacterial cultures, which improves the functional properties of the product. Probiotics are live microorganisms

that have beneficial effects on health of their host. Fermented fish is an important source of nutrients, which include essential proteins, unsaturated fatty acids, vitamins and minerals. Viability of starter culture during the fermentation process is therefore very important (Ammor *and Mayo,* 2007).The objective of this research was to isolate, characterize (Morphological, Biochemical) and identify (Molecular level) the viable Lactic acid bacteria from the fermented fish products developed at Industrial Technology Institute in order to check the ability of the starter Lactic acid bacterial inoculums to be viable during the fermentation.

Materials and Methods

One gram of forty eight hour fermented *kelawalla* fish developed at Industrial Technology Institute was dispensed in 9 ml of 0.89 % sterilized saline and vortex for 30 seconds. Serial dilution was carried out up to 10^{-6} dilution. From each dilution tubes, 0.1 ml was pipetted out on to the solidified MRS Agar media and inoculums were spread by using a sterile spreader by following the spread plate technique. Plates were incubated at 37 ^{0}C for 48hrs. Isolation was conducted in duplicates. Purification of isolated colonies was carried out by streaking the isolated colonies on MRS plates. Purified isolates were subjected to gram staining, endospore staining and motility was investigated by hanging drop method. The morphological characteristics of isolates were noted by observing their texture, colour, size, elevation, margin and consistency. Bio chemical characterization (Indole test, Methyl red test, Voges Proskauer's test, Citrate utilization test, H_2S production test and Urease test) of the nine isolates were performed. Sugar fermentation pattern (Glucose, Lactose, Arabinose, Manitol, Sorbitol, Maltose, Dextrose, Sucrose, Fructose) of nine isolates were studied by inoculating isolates to MRS broth which was contained phenol red pH indicator and respective sugar. Tolerance of nine isolates to different concentrations of salt (3%, 6% and 9%), different temperatures (30 ^{0}C, 37 ^{0}C and 40 ^{0}C) and different pH values (2, 3 and 4) were studied. The molecular level characterization of isolates was investigated. DNA of the isolates was extracted using an in-house optimized SDS proteinaseK DNA extraction method. For the PCR, primers 1492R(5'TACGGYTACCTTGTTACGACTT-3') and 27F (5' AGAGTTTGATCMTGGCTC AG-3') were selected and for the sequencing, primers 518F (5' CCAGCAGCCGCGGTAATACG 3') and 800R (5' TACCAGGGTATCTAATCC 3') were selected. The 16S rDNA sequencing was carried out at Macrogen-South Korea and sequence alignment was carried out by Basic Local Alignment Search Tool.

Results

Total nineteen organisms were isolated and based on the colony morphology, nine isolates were selected .Among them seven were Gram positive and two were gram negative. All the nine isolates were observed to be non spore foaming and non motile. All the nine isolates were negative for Indole, Catalase, H_2S production and citrate utilization and could ferment fructose.

Table no 1 represents the results of Staining, Motility and Biochemical tests of the isolates

Table 1: Results of Staining, Motility and Biochemical tests of the isolates

Isolate no	1	2	3	4	5	6	7	8	9
Gram Reaction	Negative Cocci	Positive Cocci	Positive Cocci	Positive Bacilli	Positive Cocci	Negative Cocci	Positive Cocci	Positive Cocci	Positive Cocci
Endospore Stain	-	-	-	-	-	-	-	-	-
Motility	-	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-
MR	-	-	+	-	+	-	+	+	-
VP	-	+	-	-	-	-	-	-	-
Catalase	-	-	-	-	-	-	-	-	-
H_2S.	-	-	-	-	-	-	-	-	-
Citrate uti.	-	-	-	-	-	-	-	-	-
Urease	+	-	-	+	+	+	+	-	+

All the isolates show optimum tolerance to 37 ^{0}C and 40 ^{0}C. The optimum tolerance pH was 3-4. At pH 2 the growth is weak. All of the isolates were showing the optimum growth at 6 % salt concentration. However they can tolerate 3 % and 9 % salt. By molecular level identification, the isolates were identified as *Pediococcusacidilactici* (AB680157.1), *Lactococcuslactis* (CP006766.1), *Lactococcuslactis* (KJ690920.1) and*Weissellaparamesenteroides* (HQ009793.1).Table no 2 represents the results of

molecular level identification of isolates. However, isolate no 1, 2 and 9 was not able to confirm by 16S r RNA sequencing.

Table 2: Molecular level identification of isolates

Isolate No:	**Organism**	**Accession No.**
2	Pediococcus acidilactici	AB680157.1
3	Lactococcus lactis	CP006766.1
4	Weissella paramesenteroides	HQ009793.1
5	Lactococcus lactis	KC754747.1
7	Lactococcus lactis	KJ690920.1
8	Lactococcus lactis	CP006766.1

Discussion

This research provides strong evidence on survival of culturable Lactic Acid Bacteria in the fermented fish during and after the fermentation. Since, *Weissella paramesenteroides* was not a bacterium in starter inoculums, it can be assumed that population of starter culture was changed and improved during fermentation. Furthermore *Weissella spp* was reported to be use as adjunct culture during probiotic fermentation.

Conclusion

Pediococcus *acidilactici, Lactococcus lactis* and *Weisella paramesenteroides* are the starter cultures that have the ability to be viable during the fermentation.

Reference

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