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Characterization of Beneficial and Spoilage Microflora of Onion (*Allium cepa* L.)

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Abstract

Onion (*Allium cepa* L.) is a highly nutritious vegetable with medicinal properties and is used for culinary purpose in most dishes. The post-harvest losses in onion account upto 50 per cent, mostly by microbial spoilage. The present study deals with the native microflora associated with Bhima Red onion variety; lactic acid (LA) bacteria, spoilage bacteria and fungi were isolated. The biochemical tests exhibited diversity of LAB and spoilage bacteria. The molecular characterization revealed LAB 16 and LAB 19 as *Levilactobacillus brevis*, a beneficial microorganism. *Paenibacillus polymyxa*, *Bacillus spizizenii*, *Bacillus subtilis*, *Aspergillus tamarii*, *Aspergillus welwitschiae* as dominant spoilage microorganisms. The spoilage microorganisms were confirmed as phytopathogens by cellulase and pectinase activity. All the spoilage microorganisms were positive for cellulase and pectinase activity. *Aspergillus tamari* showed the highest pectinase (0.35 U/ mL/ min) and cellulase activity (0.21 U/ mL/ min); crude enzyme extract of pectinase (37.2 mg/ mL) and cellulase (15.21 mg/ mL). *Paenibacillus polymyxa* exhibited the highest pectinase (0.03 U/ mL/ min), cellulase activity (0.02 U/ mL/ min). The crude enzyme extract of pectinase (9.48 mg/ mL) and cellulase (5.48 mg/ mL) was the highest in *Bacillus spizizenii*.

Keywords : Lactic acid (LA) bacteria, Spoilage fungi, Spoilage bacteria

Onion (*Allium cepa* L.) belongs to the family *Alliaceae*, which includes garlic, shallot and leeks. The global production of onion bulbs is estimated to be approximately 88.52×10^6 tons of which 21.40×10^6 tons are produced in India (FAOSTAT, 2017). It is estimated that 40 to 50 per cent of the stored onion never reaches to the consumers because of various types of losses. In tropical countries, such losses may be higher than the estimates (Salunkhe and Desai, 1984). These losses comprises of physiological loss in weight (PLW) *i.e*., moisture losses and shrinkage (30-40%), rotting (10-12%) and sprouting (8-10%). Onion leaves and bulbs are subjected to various spoilage problems, *viz*., physical, chemical and microbial; the latter being the most serious particularly bacterial and mold growth (Kocal *et al*., 2008). Onion spoilage leads to several health problems as spoilage is due to many pathogenic microorganisms such as *Streptococcus pyogens*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus saprophyticus*, *Bacillus lensus*, *Bacillus subtilis* and fungal species includes *Aspergillus niger*, *Aspergillus flavus*, *Fusarium oxysporum* and *Penicillium digitatum,* etc.

Onion is also associated with microorganisms that are considered beneficial. A few lactic acid (LA) bacterial species like *Lactobacillus zymae*, *L. malefermentans* and *L. plantarum* and also acetic acid (*Acetobacter pasteurians*, *A. orientalis*) and citric acid bacteria

(*Citrobacter* sp.) are involved in onion fermentation. Some of the yeast species *viz*., *Candida humilis*, *Saccharomyces boulardii* have been reported as dominant yeasts by Nkem *et al*. (2020).

The pathogens such as *Pseudomonas* sp., *Staphylococcus* sp. and *Erwinia* sp., were identified with the crop (Dogondaji *et al*., 2005) and this often reduces the germination and yield of the onion crop. The attacks by pathogen results in decreased photosynthesis and as a consequence yield loss is experienced (Berger *et al*., 2007). The pathogen infection often leads to plant death, the development of chlorotic and necrotic lesions and a decrease in photosynthetic assimilate production (Frazier and Westhoff, 2005; Banwart, 2001 and Sang *et al*., 2014). This work aims to isolate, identify and characterize microorganisms that are associated with spoilage of onions and to categorize the pathogenic microorganisms associated with spoilage of onions.

Material and Methods

Sample Collection

The Bhima Red variety of onions were procured directly from godown, in Yeshwanthpur, Bengaluru, Karnataka. The samples were separated and kept in sterile polythene bags and taken to the laboratory for further analysis.

Enumeration and Isolation of Microorganisms

The standard plate count method was followed to enumerate and isolate microorganisms from spoilt onion samples (Williams and Cross, 1971). De Man, Rogosa and Sharpe (MRS) (De Man *et al*., 1960), nutrient agar (Micheal and Frank, 1978 and potato dextrose agar medium (Baird *et al*., 2015) were used to isolate and enumerate lactic acid (LA) bacteria, spoilage bacteria and fungi respectively. LA bacteria and spoilage bacterial plates were incubated at 30°C for 48 h, while fungal plates were incubated at 25°C for 96 h. All the bacterial isolates were identified based on colony characteristics, morphological and biochemical reactions as described in Bergeys' Manual of Determinative Bacteriology (9th edition). The fungal isolates were observed under a microscope using a wet mount method. Slides were prepared using mold isolates and observed for spore arrangement, by comparing with the diagram and structure of known fungi using the identification keys of Ellis *et al*. (2007); Campbell *et al*. (2013); Lohit & Krishna (2021) and Rashmi & Suvarna (2022).

Screening of Spoilage Bacteria and Fungi

Spoilage bacteria and fungi were grown on nutrient agar and potato dextrose agar medium, respectively. The growth of spoilage bacteria and fungi at 12, 24 and 36 h intervals were qualitatively noted down. This qualitative screening was carried out to identify potential phytopathogens in onions.

DNA Extraction and Sequencing.

DNA extraction was carried out using the JETM DNA isolation kit according to the manufacturer's protocol. DNA quality and concentration were measured using a NanoDrop 1000 spectrophotometer. Five microliters of DNA samples were sent to the Barcode biosciences for 16S rRNA gene (16S), fungal internal transcribed spacer region (ITS) library preparation and sequencing. Samples were multiplexed using a dual-indexing approach and sequenced using an Illumina MiSeq with MiSeq Reagent Kit v3 (2 × 300 bp). All PCR procedures, primers and Illumina sequencing details are described in Comeau *et al*. (2016) and Yurgel *et al*. (2017). The sequence analysis and homology search sequence results were analyzed using online software from National Centre for Biotechnology Information (NCBI).

Pectinase and Cellulase Activity Assay by DNS Method

The enzyme assay was done by DNS (di-nitrosalicylic acid) method to determine the amount of reduced sugar (glucose equivalent) released. A standard graph was plotted with various dilutions of glucose solution, measuring OD at 540 nm. For analysis of sugar reduction, reaction mixtures were prepared and added to test samples, which were incubated in a 50° C water bath for 30 min. DNS reagent was added to each tube and again incubated in a 100°C boiling water bath for

10 min. OD was measured at 540 nm (Wanmolee *et al*., 2016). The concentration of substrate released was measured using the glucose standard curve. All tests were conducted in triplicates. This test was carried out to confirm phytopathogenicity of spoilage bacteria and fungi.

Protein Estimation and Enzyme Activity by Lowry's Method

Protein estimation was done by Lowry's method. A standard graph was prepared using graded concentrations of bovine serum albumin stock solution and OD was measured at 660 nm. For analysis of protein, reaction mixtures were prepared and added to test samples, which were incubated in dark for 30 min. The OD was measured at 660 nm and the graph was prepared to estimate protein concentration in each test sample of crude enzyme (Lowry *et al*., 1951). Enzyme activity calculated for each sample is expressed as 'U/ mL/ min''.

Statistical Analysis

The Opstat 2.0 was used for the data analysis, statistically significant different groups were calculated with the One-way Analysis of variance (ANOVA). The Duncan test was used for multiple comparisons and the level of statistical significance was set at $p \leq 0.05$ (Panse and Sukhatme, 1967).

Results and Discussion

This study was mainly focused to identify native beneficial and spoilage microflora associated with onion. The identification of native microflora helps us to clearly understand the major opportunistic pathogens, human pathogens if present and beneficial LA bacteria; which have been used from days primitive to control various spoilage organisms. The results of the current study are presented and discussed here.

Enumeration and Isolation of Microorganisms

Lactic acid (LA) bacteria have been found in diverse nutrient rich environments, including milk, dairy products, plants, soil, meat and meat products. Among beneficial microbes, LA bacteria have many biopreservation metabolites such as LA, organic acid, exopolysaccharide, phenolic compounds and so on. For LA bacteria, morphological and biochemical characteristics were analyzed and they varied in pigmentation, colony size and shape, elevation, margin, shape and Gram's reaction (Table 1). LA bacterial genera have been reported to be present in onions (Nannu & Chandra, 2015; Jung *et al*., 2012 and Kim *et al*., 2004).

Spoilage bacteria and fungi are present abundantly due to their vigorous growth and capacity to utilize a wide range of substrates. As many as 15 spoilage bacteria and six fungal isolates (six) were selected and pure cultured. Spoilage bacteria and fungi varied in morphological attributes and results have been represented in Table 2 and 3, respectively. Spoilage bacteria and fungi have been found to be present in spoilt onion bulbs (Nkem *et al.*, 2020 and Salami *et al*., 2019).

Screening of Microorganisms for Growth Potential and Antimicrobial Activity

A total of 15 spoilage bacteria and six spoilage fungi were selected, pure cultured and stored for further characterization. Screening of spoilage microorganisms was carried out based on their growth potential at different intervals (12, 24 and 36 h) and the growth of these microorganisms was measured based on qualitative parameters. The growth of spoilage microorganisms on agar media were represented as dense, medium, light, very little and no growth (Table 4). The LA bacteria isolates (26) were screened under *in vitro* condition for their antimicrobial activity against spoilage microorganisms and LA bacteria isolates (six) were selected for further work (Unpublished data).

Biochemical Characteristics of Screened LA and Spoilage Bacteria

The screened LA bacterial isolates were subjected to biochemical tests (Table 5). Spoilage bacteria, obtained based on vigorous growth were subjected to biochemical tests (Table 6).

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TABLE 1
Morphological and biochemical characteristics of LA bacteria

LA bacteria	Morphological and biochemical observations					
	Pigmentation	Colony size and shape	Elevation	Margin	Shape	Gram's reaction
LAB1	Creamish white	Small circular	Raised	Entire	Rods	+
LAB2	Dull white	Small circular	Raised	Entire	Rods	+
LAB3	Creamish white	Small circular	Raised	Entire	Cocci	+
LAB4	Creamish white	Small circular	Raised	Entire	Cocci	+
LAB5	Creamish white	Small circular	Raised	Entire	Cocci	+
LAB6	Dull white	Small circular	Raised	Entire	Rods	+
LAB7	Creamish white	Small circular	Raised	Entire	Cocci	+
LAB8	Dull white	Small circular	Raised	Entire	Cocci	+
LAB9	Dull white	Small circular	Flat	Entire	Rods	+
LAB10	Bright white	Small circular	Raised	Entire	Cocci	+
LAB11	Dull white	Small circular	Raised	Entire	Cocci	+
LAB12	Bright white	Small circular	Raised	Entire	Cocci	+
LAB13	Bright white	Small circular	Raised	Entire	Cocci	+
LAB14	Bright white	Small circular	Raised	Entire	Cocci	+
LAB15	Bright white	Small circular	Raised	Entire	Cocci	+
LAB16	Bright white	Small circular	Raised	Entire	Rods	+
LAB17	Translucent	Irregular	Flat	Entire	Cocci	+
LAB18	Dull white	Irregular	Raised	Undulate	Cocci	+
LAB19	Dull white	Irregular	Raised	Entire	Rods	+
LAB20	Dull white	Irregular	Raised	Entire	Cocci	+
LAB21	Dull white	Irregular	Raised	Entire	Cocci	+
LAB22	Dull white	Irregular	Raised	Entire	Cocci	+
LAB23	Dull white	Irregular	Raised	Entire	Cocci	+
LAB24	Dull white	Irregular	Raised	Undulate	Cocci	+
LAB25	Dull white	Small circular	Umbonate	Entire	Cocci	+
LAB26	Dull white	Small circular	Raised	Undulate	Cocci	+

Note :- '+'- positive, '-' – negative.

Sequence Analysis and Homology Search

In the present study, selected spoilage bacteria were identified as *Paenibacillus polymyxa*, *Bacillus spizizenii* and *B. subtilis*; spoilage fungi as *Aspergillus tamarii* and *A. welwitschiae*. LA bacteria isolates (two) selected based on antimicrobial attributes were identified as *Levilactobacillus brevis* (Table 7).

Protein Estimation and Enzyme Activity

The phytopathogens if they have to thrive, must be able to ramify plant cell wall constituents such as cellulose microfibrils, glycans, pectin and polysaccharides. Filamentous fungi are excellent sources of extracellular enzyme production. In the present study, *Aspergillus tamarii* showed the highest

TABLE 2

Morphological and biochemical characteristics of spoilage bacteria

Spoilage bacteria	Morphological and biochemical observations					
	Pigmentation	Colony size and shape	Elevation	Margin	Cell Shape	Gram's reaction
SB1	Dark Red	Small circular	Convex	Entire	Rods	-
SB2	Translucent	Small circular	Flat	Entire	Cocci	-
SB3	White	Irregular	Pulvinate	Curled	Cocci	+
SB4	Bright white	Small circular	Convex	Entire	Rods	+
SB5	Creamy white	Small circular	Convex	Entire	Rods	+
SB6	Creamy white	Irregular	Umbonate	Undulate	Rods	+
SB7	Bright yellow	Small circular	Convex	Entire	Cocci	-
SB8	Pinkish white	Irregular	Flat	Lobate	Cocci	+
SB9	Bright white	Small circular	Flat	Entire	Rods	+
SB10	Creamy white	Irregular	Umbonate	Curled	Rods	+
SB11	Creamy white	Irregular	Umbonate	Undulate	Rods	+
SB12	Transparent	Small circular	Flat	Entire	Rods	+
SB13	White	Irregular	Umbonate	Undulate	Rods	+
SB14	White	Irregular	Umbonate	Curled	Rods	+
SB15	Dark red	Small circular	Convex	Entire	Rods	-

Note :- '+'- positive, '-' – negative.

TABLE 3

Morphological characteristics of spoilage fungi

Spoilage fungi	Morphological observations					
	Texture	Colour	Elevation	Margin	Hyphae	Conidia / Spore
SF1	Cottony	Creamy white	Raised	Irregular	Aseptate	No sporulation
SF2	Cottony	Green with yellow and white margin	Umbonate	Circular	Septate	Club shaped
SF3	Cottony	Light grey with white margin	Raised	Circular	Aseptate	No sporulation
SF4	Cottony	Dark Grey with white margin	Umbonate	Velvety	Septate	Club shaped
SF5	Cottony	Whitish grey	Raised	Circular	Septate	No sporulation
SF6	Velvety	White with pale green border	Raised	Circular	Aseptate	No sporulation

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TABLE 4

Growth of spoilage bacteria and fungi at different time intervals

Spoilage bacteria	Growth, after h of incubation		
	12	24	36
SB1	M	M	M
SB2	M	M	M
SB3	L	M	M
SB4	VL	VL	VL
SB5	M	M	M
SB6	D	D	D
SB7	D	M	D
SB8	VL	VL	VL
SB9	M	M	M
SB10	D	D	D
SB11	D	D	D
SB12	M	M	M
SB13	L	L	L
SB14	M	M	M
SB15	D	M	M
		Growth	
Spoilage fungi	12 h	24 h	36 h
SF1	VL	VL	VL
SF2	D	D	D
SF3	L	M	M
SF4	D	D	D
SF5	M	M	M
SF6	L	M	M

Observations represented are the mean of three replicates

D : Dense VL : A little growth M : Medium
N : No growth L : Light

pectinase and cellulase activity and production. *Paenibacillus polymyxa* showed the highest pectinase and cellulase activity. The crude enzyme extract of pectinase and cellulase was the highest in *Bacillus spizizenii* (Fig. 1 and 2). Parallel results in cellulase and pectinase activity and protein concentration were found in spoilage organisms (Sagar *et al*., 2019 and Oyeleke *et al*., 2012).

This study reveals that onions possessed diversified microflora ranging from spoilage to beneficial organisms. Beneficial organisms mainly included LA bacterial isolates, which were capable of producing

TABLE 5

Biochemical characteristics of LA bacteria

Biochemical test	Isolates					
	LAB5	LAB10	LAB15	LAB16	LAB19	LAB23
Arabinose	-	+	-	+	+	-
Glucose (acid)	+	+	+	+	+	+
Glucose (gas)	+	+	+	+	+	+
Lactose	-	-	-	-	-	-
Sucrose	+	+	+	+	+	-
Maltose	-	+	+	+	+	-
Mannose	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-
Esculin	-	-	-	-	-	-
Fructose	+	+	+	+	+	+
Galactose	+	+	+	+	+	+
Mannitol	-	+	-	-	-	+
Catalase test	-	-	-	-	-	-

Note :- '+'- positive, '-' – negative.

TABLE 6

Biochemical characteristics of spoilage bacteria

Biochemical test	Isolates		
	SB6	SB10	SB11
IMViC test			
Indole	+	-	+
Methyl Red	+	+	+
Vogues Proskauer	-	+	+
Citrate utilization	-	-	-
Carbohydrate fermentation test			
Glucose (acid)	+	+	+
Glucose (gas)	-	+	+
Maltose	-	-	-
Sucrose	+	+	-
Mannose	+	+	-
Fructose	-	+	+
Mannitol	+	+	-
Gelatin Hydrolysis	-	-	-
Catalase test	+	+	+

Note :- '+'- positive, '-' – negative.

TABLE 7

Identification of microorganisms isolated from Bhima Red onion variety

Isolate code	Organism identified	Accession no.	Closest type strain in the NCBI database	Sequence length	Sequence similarity
LAB 16	*Levilactobacillus brevis* strain UASB MIC_001	ON377356	*Levilactobacillus brevis* strain ATCC 14869	1485 bp	99.67
LAB 19	*Levilactobacillus brevis* strain UASB MIC_002	ON377357	*Levilactobacillus brevis* strain ATCC 14869	1448 bp	98.78
SB 6	*Paenibacillus polymyxa* strain UASBMIC_003	ON377358	*Paenibacillus polymyxa* strain DSM 36	1463 bp	99.43
SB 10	*Bacillus spizizenii* strain UASBMIC_004	ON377359	*Bacillus halotolerans* strain DSM 8802	1449 bp	99.32
SB 11	*Bacillus subtilis* strain UASBMIC_005	ON377360	*Bacillus subtilis* strain IAM 12118	1450 bp	97.87
SF2	*Aspergillus tamarii* strain UASBMIC_F1	ON377371	*Aspergillus tamarii* NRRL 20818	502 bp	99.09
SF4	*Aspergillus welwits chiae* strain UASB MIC_F2	ON377372	*Aspergillus welwitschiae* CBS 139.54	618 bp	99.24 -

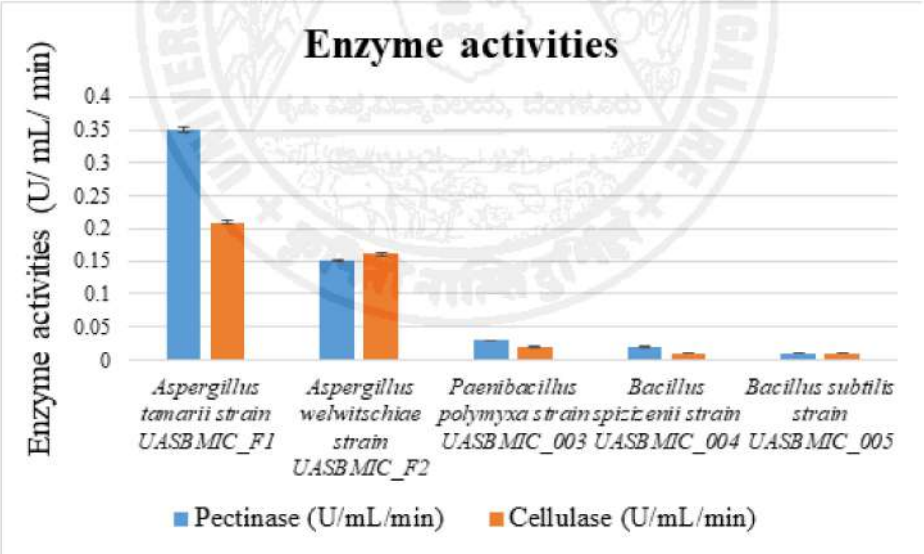

Fig. 1 : Enzyme activities of the spoilage organisms

antimicrobial metabolites such as LA, organic acid, exopolysaccharide, diacetyl, H_2O_2, phenolic compounds and so on (Unpublished data). Spoilage organisms produced extracellular enzyme and crude protein sufficient enough to hydrolyze cellulose and pectin, finally causing spoilage of onion. A clear understanding of each microorganism can help us to mitigate losses in onion and also reduce the health risk associated with spoilage microorganisms. Further, work on the commercialization of promising LA bacteria to overcome spoilage of bulbs can be concentrated on a priority basis.

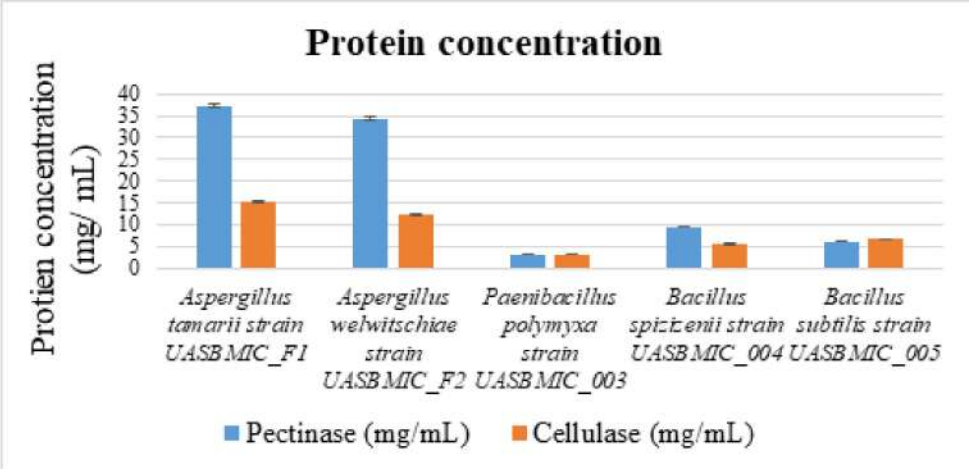

Fig. 2 : Protein concentration of crude enzyme extract (BSA standard graph) of the spoilage organisms

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