



ISOLATION, IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF *SERRATIA MARCESCENS* STRAIN NMRL65 AN OPPORTUNISTIC HUMAN PATHOGEN FROM THE SPOILED COCONUT

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Received on: 28.03.2020

Revised on: 20.04.2020

Accepted on: 30.04.2020

Abstract

Microbes are the common cause of food spoilage and are widely distributed in the environment. Fruits and vegetables provide ideal nutrient conditions for the survival and growth of many types of bacteria. Bacteria responsible for food spoilage produce extracellular hydrolytic enzymes which bring about the deterioration of the food products. Coconut flesh is highly nutritious and rich in saturated fat, sugar and potassium that have wide range of health benefits. Fresh coconut flesh is often spoiled by red pigment producing bacterium *Serratia*. The present study was therefore undertaken and a red pigment producing opportunistic human pathogen *Serratia marcescens* strain NMRL 65 from spoiled coconut was isolated, identified and characterized for specific genes. Also its ability to produce multiple hydrolytic extracellular enzymes like chitinase, lipase and protease was investigated and reported.

Keywords

Food spoilage bacteria, *Serratia marcescens*, opportunistic human pathogen, lipase, protease, coconut,

1. Introduction

Many countries around the world are facing food insecurity due to increasing global human population. The second goal of United Nations Sustainable Development is to end hunger in all its forms by 2030 and to achieve food security. Large proportions of food products are spoiled during the food supply chain by food spoilage microorganisms. One third of the food products produced for human population are either spoiled or wasted according to Food and Agriculture Organization of the United Nations 2011 report. Food spoilage is broadly defined as a change in the quality of food items that makes it undesirable for human consumption, due to spoilage indicators such as bad odour, texture and appearance [1]. The cause of food spoilage is a complex process broadly grouped as microbiological, chemical and physical [2]. Microorganisms responsible for food spoilage are bacteria, fungi and yeast. Microbes are able to survive in different environmental conditions like high salt, sugar, acidic and alkaline. Fruits with high water and sugar content are easily spoiled by microbes. Fresh coconut flesh is easily spoiled by bacteria, fungi and yeast due to its sugar, fat and water content.

The *Serratia* genus are Gram negative, facultative anaerobic bacteria, which grow in a broad range of temperatures and substrates, including plant surfaces, soil, water, and food products such as fruit juices, coconut and fish. *Serratia marcescens* and *S. rubidaea* is commonly found as a contaminant in wet coconut as a pink or red colour colonies. The red pigment produced by *S. marcescens* is prodigiosin. Prodigiosin is produced in the later stages of bacterial growth, although it doesn't have any physiological role in producing organisms, it's reported to have antibacterial, antifungal, antiprotozoal and antitumor properties [3].

Serratia marcescens produces wide range of extracellular hydrolytic enzymes such as chitinase, protease and lipase, due to this it is able to degrade and spoil edible food items rich in protein and fat. The presence of *S. marcescens* in an edible food items like coconut should be considered as a cause of concern because it's classified as an opportunistic human pathogen. About 2% of nosocomial infections of the bloodstream, lower respiratory tract, urinary tract, surgical wounds and skin and soft tissues of adult persons are caused by *Serratia*[4].

Quorum sensing allows bacterial populations to communicate and coordinate group behaviour and commonly is used by pathogens in disease and infection processes. Quorum sensing systems controls a diverse range of cell density dependent factors such as antibiotic, biosurfactant production, extracellular polysaccharide, biofilm differentiation, cell division and pathogenicity. *Serratia* utilizes two types of quorum sensing signalling molecules: N-acyl homoserine lactones (AHLs, AI-1) and AI-2 type signalling molecules. The two key enzymes in AI-2 signalling molecules in *Serratia* is S-ribosylhomocysteinase (*LuxS*) and S-adenosylhomocysteine nucleosidase (*Pfs*) are responsible for biosynthesis of signalling molecules, which contribute to virulence factors of the human opportunistic pathogen *S. marcescens*[5].

To detect and identify the presence of *S. marcescens*-an opportunistic pathogen in edible food products like coconut, using reliable, rapid and internationally accepted test methods is of great importance. In this study a red pigment producing bacterium was isolated from spoiled coconut and identified using *S. marcescens* species specific polymerase chain reaction as *S. marcescens* strain NMRL65. The genes coding for serrawettin biosurfactant production, chitinase, serrapeptidase, S-ribosylhomocysteinase (*LuxS*) and S-adenosylhomocysteine nucleosidase (*Pfs*) were also detected in *S. marcescens* strain NMRL65 by polymerase chain reaction.

2. MATERIALS AND METHODS

All the microbiological media used were procured from Hi-Media Laboratory Pvt., Ltd. (Mumbai, India). Molecular biology reagents were procured from Thermo Fisher Scientific (USA). Water used throughout was of Milli-Q grade.

2.1. Sample Collection

Spoiled wet coconut with pinkish red colour discoloration was collected from D2/6, Residential Quarters of Naval Materials Research Laboratory, Ambernath.

2.2 Isolation of bacteria from pinkish red discoloured spoiled coconut

The pinkish red discoloured spoiled coconut was swabbed using sterile wooden cotton swab (Hi-Media Labs Pvt., Ltd. Mumbai) and inoculated in 50 ml of sterile Nutrient Broth in 250 ml conical flask and incubated in an rotary shaker (Orbitek Shaker, Scigenics Pvt. Ltd., Chennai, India) at 28°C for 48 hour. After 48 hours 10µl of reddish nutrient broth was inoculated in prepared sterile Nutrient Agar (NA) petri-plates and quadrilateral streaking was done and incubated in microbiological incubator at 28°C for 48 hours to isolate food spoilage bacteria. The red pigmented colony was selected and detailed morphological, biochemical and molecular biology characterization was done. The red pigmented bacterial

isolate was labelled as strain NMRL65 and stored in glycerol stock at - 80°C for further characterization [6].

2.3 Identification of red pigment producing bacterium *S. marcescens* strain NMRL65 from spoiled coconut using *S. marcescens* species specific polymerase chain reaction

Total DNA extraction of bacterial strain NMRL65 was performed using DNeasy ultraclean microbial kit (Qiagen, Germany). The strain NMRL65 was further identified till the species level by using primers specially designed for the identification of *S. marcescens* based on the 16S rRNA sequence alignment of different environmental isolates [7]. The nucleotides sequence of *S. marcescens* species specific forward primer are Smar16SV89-108 -5'-GGGAGCTTGCTCACTGGGTG-3' and reverse primer Smar16SWR 499-471-5'-GCGAGTAACGTCA GTTGATGAGCGTATTA-3'. The PCR amplification was performed in the total volume of 25µl in 200µl capacity thin wall PCR tube consisting of 7.5 µl of PCR grade water, 12.5 µl of 2X PCR master mix (Thermo Scientific), 1 µl (10 picomoles) of each primer, and 3 µl of template DNA (50 picomoles). Amplification was performed in a thermal cycler Mastercycler egradient PCR machine (Eppendorf, Hamburg, Germany). The temperature profile for PCR was kept, 95°C for 3 min, 94°C for 0.5 min, 69°C for 1min, 72°C for 0.45 min, 35cycles and final extension at 72°C for 10 min. The PCR product was then electrophoresed in 1.2% agarose and documented using G:Box gel document system (Syngene, USA).

2.4 Morphological and Biochemical identification of *S. marcescens* strain NMRL65

The morphological characterizations of the strain NMRL65 was carried out by Gram Staining and hanging drop method for motility. To determine the lipase activity the strain NMRL65 was streaked on lipase medium (peptone 10g, sodium chloride 5g, calcium chloride 0.1g, Tween 80 10ml, agar 20 g dissolved in 1000 ml of distilled water). After 3 days of incubation at 28°C, the lipase activity was indicated by the powdery deposit zone surrounding the colonies. Protease enzyme was determined using skimmed milk agar (pancreatic digest of casein 5g, yeast extract 2.5g, glucose 1g, 7% skimmed milk solution 100 ml, agar 15g dissolved in 1000 ml distilled water). After 2 days of incubation at 30°C, a clear zone around the cells indicated positive proteolytic activity [8].

Phosphate solubilizing enzyme was determined by the development of a clear zone in Pikovskaya's agar medium (yeast extract 0.5g, dextrose 10 g, calcium phosphate 5g, ammonium sulphate 0.5g, potassium chloride 0.2g, magnesium chloride 0.1g, manganous sulphate 0.0001g, ferrous sulphate 0.0001g, agar 15g dissolved in 1000ml distilled water) after 2-5 days incubation of assay plates at 30°C [9].

25 Detection of *chiA* gene coding for chitinase A enzyme in *S. marcescens* strain NMRL 65 by PCR

The *ChiA* gene coding for chitinase A enzyme in strain NMRL 65 was detected using reported *chiA* specific primers: 5'-GATATCGACTGGGAGTTCCC-3' (forward; corresponding to nucleotide positions 931-950 of *S. marcescens* X03657) and 5'-CATAGA AGTCGTA GGTCATC-3' (reverse: positions 1180-1161) flanked a 225 bp fragment[10]. The PCR amplification was performed in the total volume of 25µl in 200µl capacity thin wall PCR tube consisting of 7.5 µl of PCR grade water, 12.5 µl of 2X PCR master mix (Thermo Scientific), 1 µl (10 picomoles) of each primer, and 3 µl of template DNA (50 picomoles). Amplification was performed in a thermal cycler Mastercycler egradient PCR machine (Eppendorf, Hamburg, Germany). The temperature profile for PCR was kept, 95° C for 3 min, 95° C for 0.25 min, 58° C for 1min, 72° C for 0.25 min, 35cycles and final extension at 72° C for 10 min. PCR products were visualized by agarose gel electrophoresis and documented using G:Box gel document system (Syngene, USA).

26 Detection of Serrapeptidase gene in *S. marcescens* strain NMRL 65 by PCR

The serrapeptidase gene was amplified in strain NMRL 65 using specific forward Ser pep (L): 5'-GGAAGCTTCATATG CAATCTACTAAAAAGGCAAT-3' and reverse [Ser pep (R): 5'- GCGAAGCTTAC ACGATAAAGTAGT GGCGACGT-3'[11]. The PCR amplification was performed in the total volume of 25µl in 200µl capacity thin wall PCR tube consisting of 7.5 µl of PCR grade water, 12.5 µl of 2X PCR master mix (Thermo Scientific), 1 µl (10 picomoles) of each primer, and 3 µl of template DNA (50 picomoles). Amplification was performed in a thermal cycler Mastercycler egradient PCR machine (Eppendorf, Hamburg, Germany). The temperature profile for PCR was kept, 95° C for 3 min, 95° C for 1.5 min, 60° C for 1min, 72° C for 1.5 min, 35cycles and final extension at 72° C for 10 min. PCR products were visualized by agarose gel electrophoresis and documented using G:Box gel document system (Syngene, USA).

27 Detection of *pfs* and *luxS* genes involved in AI-2 Dependent quorum sensing in *S. marcescens* strain NMRL 65 by PCR

The S-ribosylhomocysteine (LuxS) and S-adenosylhomocysteine nucleosidase (*Pfs*) enzymes are responsible for synthesis of signalling molecules, which contribute to virulence factors of the human opportunistic pathogen *Serratia* and other genes of unknown function via quorum sensing. The *luxS* and *pfs* gene was detected in strain NMRL 65 using primers specific for *Serratia luxS* and *pfs* gene. The primers used for *luxS* are FluxS1- 5'G CTGGAACACCTGTTTCGC-3' and RluxS2-5' ATGTAGAAACCGGTGCGG-3', the primers used for *pfs* are

Fpfs1-5' CCGGCATCGGCAAAGTCT-3' and Rpfs2 -5'-ATCTGGCCCCGGCTCGTAGCC-3'[12]. The PCR amplification was performed in the total volume of 25µl in 200µl capacity thin wall PCR tube consisting of 7.5 µl of PCR grade water, 12.5 µl of 2X PCR master mix (Thermo Scientific), 1 µl (10 picomoles) of each primer, and 3 µl of template DNA (50 picomoles). Amplification was performed in a thermal cycler Mastercycler egradient PCR machine (Eppendorf, Hamburg, Germany). The temperature profile for PCR was kept, 95° C for 3 min, 95° C for 45 s, 55° C for 0.5min, 72° C for 15 s, 30cycles and final extension at 72° C for 10 min. PCR products were visualized by agarose gel electrophoresis and documented using G:Box gel document system (Syngene, USA).

28 Detection of putative *SwrW* gene required for biosurfactant serrawettin W1 production in *S. marcescens* strain NMRL65

The *SwrW* gene which encodes putative serrawettin W1 synthase was detected in strain NMRL65 using primers SW2-F3 5'-GCG ACA AAA GCA ATG ACA AA-3') and SW2-R3 5'-GTC GGC GTA TTG TTC CAA CT-3'[13]. The PCR amplification was performed in the total volume of 25µl in 200µl capacity thin wall PCR tube consisting of 7.5 µl of PCR grade water, 12.5 µl of 2X PCR master mix (Thermo Scientific), 1 µl (10 picomoles) of each primer, and 3 µl of template DNA (50 picomoles). Amplification was performed in a thermal cycler Mastercycler egradient PCR machine (Eppendorf, Hamburg, Germany). The temperature profile for PCR initial denaturation at 95°C for 3 min, 95°C for 30s and extension 72°C for 50 sec, 30 cycles of denaturation at 94°C, 0.45 mins, annealing 55°C, 0.45 min, and extension at 72°C, 3 min; 1 cycle of final extension at 72°C, 10 min. Polymerase chain reaction (PCR) products were stored at 4°C. PCR products were visualized by agarose gel electrophoresis and documented using G:Box gel document system (Syngene, USA).

3. RESULTS AND DISCUSSION

3.1 Morphological and Biochemical characterization of *S. marcescens* strain NMRL65

The *S. marcescens* strain NMRL 65 isolated from pinkish reddish discoloured spoiled coconut (Figure 1a) was characterized by Gram staining as Gram negative rods, and hanging drop method revealed motile rods. The strain NMRL65 produced non diffusible red pigment prodigiosin in nutrient agar after 48 hours of incubation at 28°C as shown in Figure 1b. The strain NMRL 65 hydrolyzed casein in skimmed milk agar producing clear zone around colonies after 48 hours which indicated the protease enzyme production as shown in Figure 2a. This enzyme plays a role in spoilage of protein rich food items like milk [14].

Lipase assay using Tween 80 as substrate revealed the hydrolysis which produced powdery deposit around the strain

NMRL 65 colonies as shown in Figure 2b. Due to the production of lipase enzyme *S. marcescens* strain NMRL 65 was able to spoil food products which are rich in fats like coconut and milk [15].

The strain NMRL 65 was able to solubilise inorganic calcium

phosphate by producing phosphate solubilising enzyme as shown in Figure 2c. Many researchers have reported phosphate solubilising capabilities of *S. marcescens* isolated from agricultural soils [16 – 18].



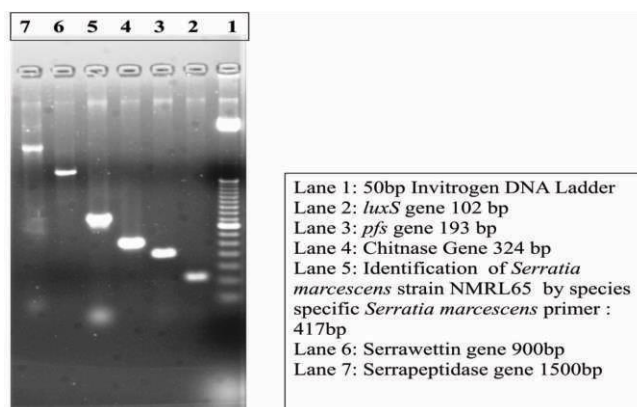
Figure 1: (a) A pinkish red discoloured spoiled fresh wet coconut (b) Isolated a red pigmented producing *S. marcescens* strain NMRL 65 in nutrient agar incubated at 28°C for 48 hours.



Figure 2: (a) Protease plate assay for *S. marcescens* strain NMRL65 in skimmed milk agar. (b) Lipase plate assay for *S. marcescens* strain NMRL65 in nutrient agar with 0.1% calcium chloride and 1% Tween 80. (c) Phosphate solubilising plate assay in Pikovskaya's agar.

3.2 Molecular Identification of *S. marcescens* strain NMRL65 by *S. marcescens* species specific PCR

The amplification of PCR product of 417 bp (Figure 3 Lane 5) from the genomic DNA of strain NMRL25 using *S. marcescens* species specific primer confirmed the strain NMRL65 as *S. marcescens*. A prodigiosin producing *S. marcescens* isolated from soil was identified by species level by using *S. marcescens* species specific primer [19].



3.3. Molecular characterization of *S. marcescens* strain NMRL65

The amplification of 225 bp PCR products in *S. marcescens* strain NMRL 65 (Figure 3. Lane 4) using reported *chiA* gene primer designed based on *S. marcescens* sequence confirmed the presence of chitinase A enzyme producing capabilities of the strain NMRL65. Many researchers have reported the presence of *chiA* gene in *S. marcescens* and its role in spoilage of chitin rich sea foods like shrimps [20, 21].

The amplification of 1500 bp PCR products in *S. marcescens* strain NMRL65 (Figure 3. Lane 7) using primer designed based on *S. marcescens* sequence confirmed the presence of Serrapeptidase enzyme capacity of this stain. Serrapeptidase is also known as miracle enzyme which can be used to dissolve blood clots and shrink varicose veins. Many researchers worldwide reported the production of serrapeptidase from *S. marcescens* [22-24].

The amplification of 900bp PCR (Figure 3, Lane 6) products in *S. marcescens* strain NMRL 65 using primer specific for serrawettin W1 synthase confirmed the biosurfactant producing property of this strain. Due to the biosurfactant producing ability *S. marcescens* is able to colonize wide range of substrates in the environment [25].

The amplification of 102bp and 193bp PCR products (Figure 3, Lane 2&3) in *S. marcescens* strain NMRL 65 using primer specific for S-ribosylhomocysteine (*LuxS*) S-adenosylhomocysteine nucleosidase (*Pfs*) sequence of *Serratia* confirmed the presence of this two key enzymes in AI-2 dependent quorum sensing signal molecules in this strain. The quorum sensing based PCR test method can be used to detect *S. marcescens* from various environments like spoiled food items and animal host. Zhu *et al.*, 2008 [12] reported detection of *luxS* and *pfs* in 27 different *Serratia* strains.

4. Conclusion

S. marcescens is widely distributed in air and cause deterioration of food products. Wet coconut rich in sugar, fat and water content is easily colonized and spoiled by this food spoilage bacterium. The *S. marcescens* species specific PCR method can be employed for rapid detection and identification of *S. marcescens* in spoiled food items.

Acknowledgement:

The authors would like to express their gratitude to the Director, NMRL, Ambarnath for his constant support and encouragement.

Conflict of Interest

The authors declare that there is no conflict of interest.

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