

Isolation and Identification of Alicyclobacillus with High Dipicolinic Acid and Heat Resistant Proteins from Mango Juice

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Abstract

Background and Objectives: Microbial spoilage of juices and industries related with Alicyclobacillus are considerable international issues. This spore-forming bacterium causes changes in juices odor and taste. The isolation and identification of Alicyclobacillus contamination in juice producing and packaging industries has an essential role in the prevention and control of this type of spoilage bacterium in HACCP (Hazard analysis and critical control points) manner.

Materials and Methods: A thermo-acidophilic, non-pathogenic and spore-forming bacterium was isolated from mango juice. Preliminary identification of the isolates was based on morphological, biochemical and physiological properties. Identification at species level was made by PCR amplification. The influence of temperature in the range of 25-65°C in the growth of bacterium and in the range of 80-120°C in spore-resistant and heat resistant proteins was investigated and compared with other spore producing bacteria.

Results and Conclusion: Phylogenetic analysis of the 16S rRNA gene sequencing indicated that the isolated strain constituted a distinct lineage in the Alicyclobacillus cluster and submitted to NCBI with access number Alicyclobacillus HRM-5 KM983424.1. The spores resisted 110°C for 3 h, and produced 28% dipicolinic acid more comparable to *Bacillus licheniformis*. Also they could produce 0.69 mg heat resistance protein after 1.5 h treatment in 100°C. The results showed that this strain could have biotechnological applications.

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1. Introduction

Fruit products such as juices are acidic, and a pasteurization treatment in the temperature range of 85–95°C should be adequate for their stabilization at ambient temperature. Such a process inactivates all nonspore-forming bacteria that are able to spoil the product. Although surviving bacterial spores do not germinate and grow under acidic conditions usually; in some cases, the bacteria can contaminate processed food products and cause economic losses of many factories [1]. Alicyclobacillus is an acidophile, spore-forming spoilage organism of concern for the fruit juice industry. The occurrence of spore-forming bacteria in low pH foods was thought to be insignificant. This bacterium was first isolated in 1967 from water sources and was named as *Bacillus*

(*B.*) *acidocaldarius*. Due to large amounts of cyclohexane fatty acids in cell membranes, the genus was called Alicyclobacillus [2,3]. However, in recent years, spoilage of acidic juice by Alicyclobacillus was recognized, and the seriousness of this situation is now being appreciated. *Alicyclobacillus* (*A.*) *acidoterrestris* has been associated with commercially pasteurized fruit juices as well as other low pH, shelf-stable products such as bottled tea and isotonic drinks [4,5]. It has been isolated from the garden and forest soils, and may be introduced into the manufacturing process through unwashed or poorly washed fruits. If spores are not destroyed by processing, they can germinate, grow, and spoil the product. Since omega-alicyclic fatty acid exist the

lipid membrane; the bacterium can deal with high temperatures and acidic conditions. Recent findings showed that the bacteria could grow at 20 to 70°C (optimum 40-60°C) and pH 2.5 to 6.0 (optimum 3-5). Alicyclobacillus has been isolated from various sources such as forest and garden soils, water, rotten foods, and fruit juices like apple juice and orange juice [6]. The growth of this bacterium is slow, and sometimes, it takes up to 5 days until the colonies can be observed in the medium. The bacterium has the ability to ferment rhamnose and lactose but not xylose, melibiose and mannose, and cannot convert nitrate to nitrite [7].

There is an assumption in the canning industry that thermophilic spore-forming bacteria are not resistant to low pH, while those bacteria that are resistant to high temperatures, they do not tolerate acidic conditions. However, studies on Alicyclobacillus concluded that some spores of this bacterium can grow in acidic conditions and high temperatures, and tolerate these conditions. Growth and multiplication of spores of the bacteria even in acidic conditions and high temperatures damage crops and the process of pasteurization (92°C in 10 s) [4,7]. Guaiacol from vanillin has a bad smell and taste which is produced by Alicyclobacillus in fruit; however, packaged juice contamination with this bacterium does not change the raw material [8]. Different methods and control of the odor of guaiacol that created by Alicyclobacillus is commonly used as synthetic flavorings in industry processed foods like roasted foods, fruit juices, chocolate ice cream, and vanilla yogurt. Additionally, Alicyclobacillus species produces heat resistant enzymes. Endoglucanase encoding gene was cloned from *A. vulcanalis* and expressed in *Escherichia coli* in terms of its potential for lignocellulose hydrolysis at high temperature and the production of biotechnology [9]. Also this bacillus produces acidic α -amylase enzyme, which is different in terms of performance with Ca^{2+} independence. As a result of high starch digestion (96.71%), it can be useful in the starch industry and sugar making process in which the repeated adjustment of pH and temperature is avoided [10]. Some strains are used in leaching of metal from coal too. In this study, the strain isolated from fruit juices and the heat stability of spores and dipicolinic acid production in the high heat treatment of spores were investigated.

2. Materials and Methods

2.1. Strain isolation

More than 28 different pasteurized products and natural juices like grapes, cherries, oranges, apples, plums and mangoes were selected. In order to enrich the bacteria, they were incubated at 37°C for 5 days. To obtain heat-resistant strains, the fruit juices were heated in water bath for 10 min at 80°C. To evaluate the resistance to pH, the samples were cultured in media with pH 3-3.5. These media included a variety of environments such as potato dextrose agar

(PDA), malt extract agar (MEA), malt extract broth (MEB), Alicyclobacillus agar and YSG agar (yeast extract 2g, glucose 1g, soluble starch 2 g, pH 3.5) [7]. Organic acids such as phosphoric acid or lactic acid were used to obtain acidic conditions. The spread plate method was used for the heated fruit juice samples; then they were incubated for 7-10 days at 40, 45, 50 and 55°C. All the isolated strains were shown to grow at different pH and temperatures.

2.2. Biochemical identification

14 strains of bacteria were selected at pH 3.5 in acidic conditions; then the strains were identified by biochemical tests like nitrate reduction, catalase and oxidase, hydrolysis of casein and starch, ability to ferment rhamnose, lactose, xylose, melibiose, mannose and the growth of strains in nutrient agar (NA), nutrient broth (NB) and salt water (NaCl 5% and 2%) [1].

2.3. Molecular identification

DNA was extracted from a 24 h bacterial culture with boiling. To identify the isolate, 16S rRNA gene was amplified using RW01AGGAGGTGATCC-AACCGCA as a forward primer and DG74 AACTGGAGGAAGGTGGGGAT as a reverse primer. The strain was identified by 16S rRNA PCR amplification, and the sequence was submitted to the NCBI Gene Bank database. An Eppendorf thermocycler instrument was used according to the temperature program (initiation 95°C for 5 min one cycle, and 95°C for 30 s for 30 cycles) [11].

2.4. Isolated strain characterizations

Spores of the isolated bacterium and other spore-producing bacteria like *B. cereus*, *Sporosarcina*, *B. subtilis* and *B. licheniformis* were isolated by plating for 6 days. Thermal resistance of the spores was examined between 80°C to 120°C. For dipicolinic acid isolation, the spores were heated at 100°C for 3h, and the UV absorption was detected at 275 nm. Dipicolinic acid was extracted from approximately 0.1 mg of spores or 0.5 ml of sporulating culture with 20 mM HCl. The suspension was diluted with 5 mM Ca^{2+} 100 mM Tris, pH 7.6, centrifuged, and the first derivative of the UV absorbance spectrum was recorded from 275 nm. Dipicolinic acid purchased from the Sigma was used as standard [12]. The heat resistant protein concentration was detected by Bradford method after different periods of time using the heat treatment at 100°C. Dipicolinic acid concentration and heat resistant protein in the isolated strain were compared with the spore-producing bacteria including *B. cereus*, *Sporosarcina*, *B. subtilis* and *B. licheniformis*.

3. Results and Discussion

Fruit-based products are a major problem for spoilage because of the unique physical properties

of fruits. *A. acidoterrestris* has been isolated from garden and forest soils and in fruit juices, and may be introduced into the manufacturing process through unwashed or poorly washed fruits [13,14].

Samples of spoilage by Alicyclobacillus spp. of pasteurized fruit juice products have increased significantly in the last few years [15]. The findings of Groenewald et al. indicated that the spores of *A. acidoterrestris* may survive in fruit juices after pasteurization treatment commonly applied in the food industry [2]. However, it is thought that contamination of fresh fruit with Alicyclobacillus during processing without proper cleaning leads to successive spoilage [4]. Results of this study indicated that among 14 isolated strains that resist acidic conditions and high temperature, one isolate from mango juice could grow on MEA at 45-55°C (optimum 45°C).

The isolated strain was a Gram-positive rod with swollen spores (Figure 1). The strain did not grow on NA and 5% NaCl; however, 2% salt solution was not sufficient to control the growth, as opposed to *B. cereus*. The strain was catalase and oxidase-positive and could hydrolyze casein and starch. The bacteria have the ability to ferment rhamnose and lactose, but not xylose, melibiose and mannose and cannot convert nitrate to nitrite. These morphological and biochemical results reveal that the isolated strain belonged to Alicyclobacillus cluster probably but these results were confirmed by molecular tests. PCR products obtained by universal 16S rRNA primers are illustrated in Figure 2. The NCBI BLAST showed that this strain was identified as 99% Alicyclobacillus and registered to NCBI with Alicyclobacillus HRM-5 KM983424.1. The phylogenetic tree showed this strain is neighbor to Alicyclobacillus strains and *B. ginsengihumi* BBN-IR-01 (Figure 2). The presence of isolated Alicyclobacillus in the samples is in agreement with other studies that reported its incidence in a wide range of fruit juices as well as in processing facilities. Groenewald et al. also reported the isolation of *A. acidoterrestris* from wash water and flume water, which increases the risk of possible recontamination by this bacterium through the water [16]. Alicyclobacillus is key to the fruit juice industry because general sterilizing techniques (92°C for 10 s) do not kill its spores [5]. Recognition of *A. acidoterrestris* as a spoilage problem in fruit juices has been increasing, and its potential to grow in a variety of fruit juices and other beverages at low pH has been established by many researchers [2,5,13,16]. Although spoilage does not cause illness, it can, however, cause consumer rejection of products, resulting in significant economic loss to the juice industry. When a product is damaged by Alicyclobacillus, the juice products develop bad odor and flavor (due to guaiacol production), but this bacterium does not change the shape of the package or bloom of the juice [6]. Not all Alicyclobacilli

produce guaiacol, and thus not all species are of spoilage concern [17].

Moreover the stability of *A. acidoterrestris* spores to survive thermal pasteurization processes requires the design of different processing techniques to pasteurization [1]. A remarkable update and overview of the most important alternative approaches to control and reduce the contamination by Alicyclobacillus spp. is reported in the paper by Tianli et al. [18]. Groenewald et al. showed that incubation temperature had a significant effect on the growth of Alicyclobacillus, and that temperature control could be used as a control measure to prevent or slow down the growth of unwanted bacteria in food [16].

The results of heat treatment of spores showed that they are heat tolerant even at 120°C for 20 min. The spores were shocked at 100°C along 3 h and cultured on MEA at 45°C for 4-7 days; then the colonies were counted. The results showed that isolated Alicyclobacillus HRM5 spores are resistant to boiling for 3 h. The thermal resistance of the spores in the spore-producing bacteria, including *B. cereus*, *Sporosarcina*, *B. subtilis* and *B. licheniformis* is compared in Table 1. Dipicolinic acid (DPA) comprises ~10% of the dry weight of spores of Bacillus species, though DPA has long been implicated in spore resistance to wet heat and spore stability [19]. The spores were collected from solid media, and their dipicolinic acids were extracted. The results are illustrated in Figure 3; as shown; the dipicolinic acid in Alicyclobacillus was almost 28% more than *B. licheniformis* and *Sporosarcina*.

Usually high temperatures for a long time denatured the proteins and DNA; however, in this case, dipicolinic acid was released and detected by standard diagram. The heat resistant proteins of the different spore producing strains determined by Bradford test are indicated in Table 2. As shown, not only Alicyclobacillus HRM5 produced more dipicolinic acid, but also produced more heat resistance proteins.

Porebska et al. found that moderate hydrostatic pressure could induce the germination of *A. acidoterrestris* spores [20]. Some process parameters, mainly temperature and low pH, strongly affected the spore germination. The ability of spores to germinate under HHP depended on the strain. The nutrients present in apple juice probably promoted the germination of *A. acidoterrestris* spores after pressurization using moderate HHP. The process of DPA release from the spores depended on the strain, pressure and temperature.

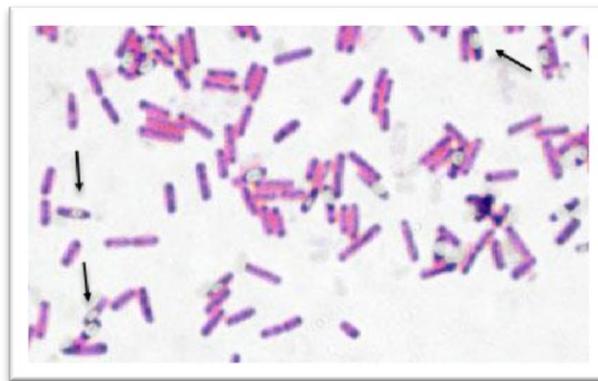
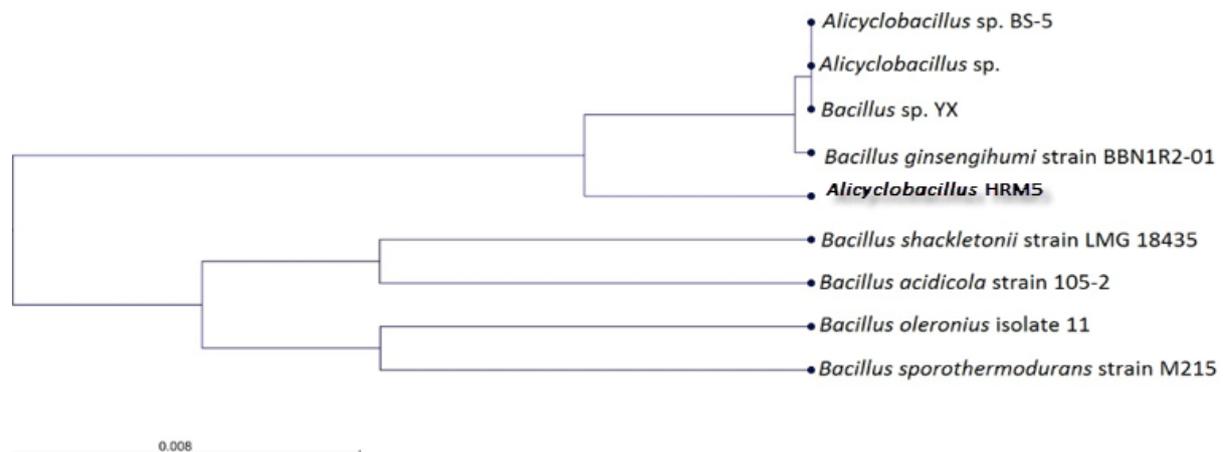
The amount of DPA released correlated to the amount of germinated *A. acidoterrestris* spores [20]. Therefore, the spores of this isolated bacterium that release high amount of DPA and heat resistant protein are a good candidate for biotechnological application, especially in drug delivery or bioleaching in acidic conditions.

Table 1. The resistance of spores to heat (100°C) by time; the table compares the heat resistance temperature of 100°C during 3h in the spore-producing bacteria by counting the colonies in MEA after 3-7 days at 45°C (0.1 ml in dilution of 10⁻³).

	0.5 h	1 h	1.5 h	2 h	2.5 h	3 h
<i>Alicyclobacillus</i> HRM-5	Many	Many	Many	29	14	4
<i>B. licheniformis</i>	Many	45	12	0	0	0
<i>B. cereus</i>	Many	75	2	0	0	0
<i>B. subtilis</i>	Many	Many	52	1	0	0
<i>Sporosarcina</i>	Many	Many	78	10	2	0

Table 2. The protein concentration (mg) detected by Bradford method at different times after heat treatment at 100°C.

	0.5 h	1 h	1.5 h	2 h	2.5 h	3 h
<i>Alicyclobacillus</i> HRM-5	0.11	0.28	0.69	0.64	0.14	0
<i>B. licheniformis</i>	0.15	0.45	0.33	0	0	0
<i>B. cereus</i>	0.2	0.37	0.68	0.56	0	0
<i>B. subtilis</i>	0.21	0.47	0.52	0	0	0
<i>Sporosarcina</i>	0.13	0.56	0.47	0	0	0

**Figure 1.** Gram staining of isolated heat resistant strains. Arrows show the spores of this bacterium.**Figure 2.** The PCR result of 16S rRNA gene and phylogeny tree for the isolated strain; constructed by CLC software

4. Conclusion

This study focused on the isolation of *Alicyclobacillus* from fruits, and characteristics of its spores. Growth and multiplication of spores of the bacteria even in acidic conditions and high temperatures damage crops and the process of pasteurization. Spores of this bacterium are good candidates for drug delivery, dipicolinic acid production,

and leaching of metals in acidic conditions. The heat resistant protein from this strain might also be interesting since the isolate hydrolyzes starch and casein, and probably has heat resistant amylase and protease. So besides food spoilage, it has a lot of applications in biotechnology, and is worth to work more on the spores of this microorganism.

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6. Conflict of interest

This article does not contain any studies with human or animal subjects. The authors have no conflict of interest to declare.

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