VNU Journal of Science: Natural Sciences and Technology, Vol. 32, No. 1S (2016) 313-320

# Identification of a New *Candida Tropicalis* Yeast Strain Possessing Antagonistic Activity Against the Spoilage Bacteria Isolated From the Fermented Vegetable Products

Nguyen Thi Thanh Mai[1], Mai Thi Dam Linh[2,*], Nguyen Phuong Thu[2], Tran Van Tuan[2]

[1]*Centre for Experimental Biology, National Center for Technological Progress, Le Thanh Tong, Hoan Kiem, Hanoi, Vietnam*
[2]*Department of Microbiology, Faculty of Biology, VNU University of Science, 334 Nguyen Trai, Thanh Xuan, Hanoi, Vietnam*

Received 08 August 2016
Revised 17 August 2016; Accepted 09 September 2016

**Abstract:** The traditional Vietnamese fermented pickles are naturally produced in presence of a microbial community including lactic acid bacteria and other microbes. Lactic acid bacteria play important roles in vegetable fermentation, whereas some yeasts have contributions to aromatic formation. However, the fermented products are sometimes spoiled by contamination of unknown bacteria during fermentation. In this study, we isolated three bacterial strains including KG 2.2, KG 2.3 and KG 2.5 in which their growth causes spoilage of the fermented pickles with formation of white biofilm layers. On basis of morphological and biochemical characteristics, these bacterial strains belong to *Bacillus* genus. The results from 16S rRNA gene sequencing indicated that the strains KG 2.2 and KG 2.5 are*Bacillus subtilis*, KG 2.3 is*B. amyloliquefaciens*. In addition, we isolated three antibacterial yeast strains from the high-quality fermentedvegetable samples. These yeasts are able to strongly inhibit growth of the spoilage bacteria. One of these strains with the best antagonistic activity named L36 was characterized as *Candida tropicalis* based on the ITS sequence of rDNA. This is the first report about *Candida tropicalis*having antibacterial activity against the food-spoilage bacteria. This yeast strain is a good candidate for preservation of traditional fermented vegetable products as a supplement.

*Keywords:* Fermented product, food-spoilage bacteria, yeast *Candida tropicalis*, antibacterial activity.

## 1. Introduction

Among the preserved foods, pickles are popular appetizer in the Southeast Asian countries, especially in Vietnam. There are two types of pickles including fermented pickles and vinegar pickles. In fact, fermented pickles are more common nowadays. Lactic acid bacteria play important roles in vegetable fermentation and some yeasts have contributions to aromatic formation [1]. However, the fermented products are suddenly spoiled by contamination of unknown bacteria during fermentation [2]. The spoilage of homemade pickles is mainly resulted from poor processing and incorrect preservation of products. The quality of processed

* Corresponding author. Tel.: 84-4-38588856
Email: linhmd@vnu.edu.vn

foodsdepends onthe input ingredients. Although, some ingredients may contribute a small part of the total value of foods, they provide substantial microorganisms.Food spoilage has been problematic for human health and fermented pickles is spoiled by molds, yeasts or bacteria [3]. Their growth causes undesirable changes in the odor, color, taste, texture, or appearance of the foods [4]. In most cases, ingredients of pickles act as potential carriers of microbial contaminants. The utilization of poor quality vegetables, fruits and spices also influences the spoilage intensity in the preserved foods [3].

Existence of yeasts in pickles was demonstrated by Etchells and coworkers in the 1950s [5]. Shortly after this report, a number of yeasts present in fermented foods were discovered.Recent studies suggest that yeasts including*Candida utilis* harbor two systems for lactic acid transformation, one of which is able to convert lactic acid to acetic acid. These organic acidshave been demonstrated to be essential for the preservation of pickles during storage [5]. Although roles of yeasts in utilizing the organic acids present in fermented productshave been evaluated so far, their other potentials for food preservation as biocontrol agent are not known yet. In this study, we have isolated three *Bacillus* strains which are able to cause pickle spoilage *in vitro*. Furthermore, we have succesfully identified a new *Candida tropicalis* strain with antagonistic activity against the *Bacillus* strains of pickle spoilage.

## 2. Materials and methods

### *2.1. Isolation and confirmation of food-spoilage bacteria*

Seven samples of spoiled pickles with the biofilm layers in surface were collected from several areas in sterilized glass bottles. A 1-ml volume ofeach sample was diluted with 9ml of sterile distilled water. This dilution step was continuously repeated until the seventh dilution. Afterwards, 0.1ml of each dilution was spread on the LB (Luria Bertani) agar plate. The plates were sealed with parafilm and incubatedat 37°C for 24 - 48 h. The bacterial colonies were selected based on morphology and purified on the LB agar plate using the three-phase streak technique. The resulting purestrains were maintained on the LB agar tubes for further studies. The bacterial strains were analyzed with Gram staining, endospore staining and biochemical tests following the common laboratory techniques. To confirm the food-spoilage capacity of the selected bacteria, these strains were added to the fresh vegetable samples together with supplementation of the lactic acid bacterium *Lactobacillus plantarum* [6] for promoting fermentation. The jars were incubated at room temperature and the spoilage phenomena were observed after one week.

### *2.2. Yeast isolation and testing the antibacterial activity of the isolated yeasts*

Tenhigh-quality fermented vegetable samples were collected from local markets in Hanoi. The samples were diluted and spread on Martin agar plates(sucrose 10g/L, peptone 5g/L, $KH_2PO_4$ 1g/L, $MgSO_4.7H_2O$ 0.5g/L, Yeast extract 0.5g/L, Rose Bengal 1/30 1ml/L, agar 16g/L, streptomycin 0.03g/L). The yeast strains were selected based on morphology with white or pink, smooth colonies.The obtained strains were purified on agar plates and cell morphology was observed under a microscope.

For antibacterial activity assay, the yeast strains were incubated at 30$^{o}$C for 24h. The cultures were centrifuged at 12,000 rpm for 30 s to collect the supernatants. The LB agar plates

were spread with the 24-h culture of the pickle-spoilage bacterial strains. Agar wells ofa diameter of 6-8 mm were made and 50 µl of the yeast supernatants were added. The plates were then incubated at 37°C for 24h. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the wells. Distilled water was used as a negative control.

*2.3. Sequencing of rDNA for the bacteria and the yeast*

The selected strains were separately cultivated in conical flasks containing 20 ml of LB broth (for bacteria) or YPD (yeast extract-peptone-dextrose) (for yeasts). The cells were harvested from 2 ml of the culture by centrifugation at 12,000 rpm for 30 s and resuspended in 50 µl of the TE buffer. The bacterial cell suspension was added 30 µl of lysozyme (10 mg/ml) and incubated for 10 min at 37ºC, whereas the yeast cell suspension was added about 150 mg of glass beads (0.5 mm-diameter type) and strongly vortexed for 30s. The mixture was added 600 µl of the extraction buffer [7] together with 2 µl of proteinase K (20 mg/ml), and incubated for 15 min at 60ºC. The tube was then added 300 µl of 3M sodium acetate (pH 5.5) and centrifuged at 12,000 rpm, 4ºC for 20 min. The genomic DNA pellet was dissolved in 50-100 µl of the TE buffer. Total RNA was eliminated from the genomic DNA samples by incubated with 3 µl of RNase (10 mg/ml) at 60ºC for 30 min.

For bacteria, the DNA products were used for PCR of 16S rRNA gene with the universal primer pair rD1 (5'-AGAGTTTGATCCTGGCTCAG - 3') / rP1 (5'-ACGGTTACCTTGTTACGACTT - 3') [7]. For yeasts, ITS of rDNA was amplified for yeast strainswith the universal primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') / ITS4 (5'-TCCTCCGCTTATTGATATGC-3') using high-fidelity Phusion DNA polymerase (Thermo Scientific) following the protocol from the manufacturer. The PCR products wereanalyzed on 1% agarose gel, purified with ANApure PCR Product & Gel Purification Mini Kit (ANABIO R&D JSC, Vietnam) and sequencedby 1st BASE company (Singapore). The obtained sequences were searched in the GenBank database and the homologous sequences were collected for phylogenetic analysis using MEGA6 software.

## 3. Results and Discussion

*3.1. The pickle-spoilage bacteria belonging to Bacillus species*

Spoiled pickle samples collected from several markets in Hanoi were prepared in serial dilutions and spread on LB agar plates. Three bacterialstrains were isolated withopaque white colonies on agar plates. All bacterial strains have long-rod cells with formation of endospores. The Gram staining tests indicated that they are Gram positive bacteria (Figure 1). With the above morphological characteristics, we could conclude that they belong to *Bacillus* genus. The detailed results are shown in Table 1.

To confirm these bacterial strains causingthe spoilage in fermented pickles, we conducted fermentation experimentsincluding 2 groups using fresh vegetable materials treated with UV light to eliminate natural microorganisms.Group 1was the control (uninfected) experiment with addition of sterile water andgroup 2 was the infected experiment withaddition ofthe isolated bacteria. Two groups were fermented with the starter culture *Lactobacillus plantarum* for 3 days. These experiments wereperformed in triplicate.

The results showed that in group 2 with addition of the spoilage bacteria, the fermented products generated an unpleasant flavor with

pH 6-7 and a white biofilm on the pickle surface after 24 h of fermentation. In contrast, in group 1 experiment, the fermented product represented good taste and high-quality appearance (Figure 2).In conclusion, all three *Bacillus* strains can cause pickle spoilage with biofilmformation on the top of the fermented products.

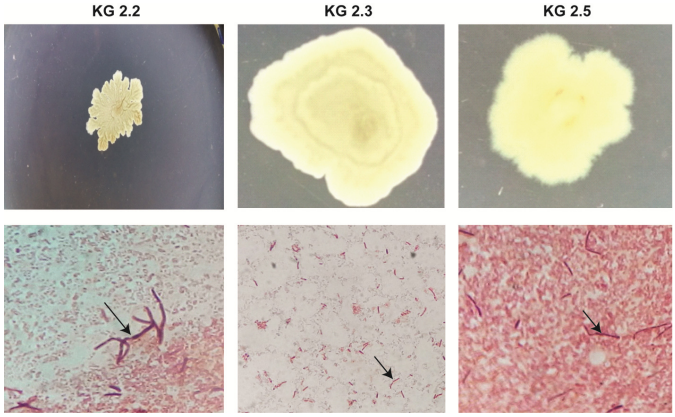


Figure 1. Morphology of colonies and Gram positive cells of three bacterial strains.
The arrows indicate the Gram positive cells and the Gram negative *E. coli* cells in pink used as control.

Table 1. Biological characteristics of the pickle-spoilage bacteria.

| **Characteristics** | **Bacterial strains** | | |
|---|---|---|---|
| | **KG 2.2** | **KG 2.3** | **KG 2.5** |
| Colony morphology | Opaque white, circular shape, viscosity and wrinkle surface | Opaque white, circular, smooth, viscous surface | Opaque white, circular shape, viscosity and wrinkle surface |
| Cell morphology | Rod, long chain | Rod, short chain | Rod, long chain |
| Gramstaining | + | + | + |
| Endospore staining | + | + | + |

To identify these bacterial strains in more detail, PCR amplification of 16S rRNA genes using the genomic DNA extracted from the pickle-spoilage bacterial strains resulted in DNA products of 1.5 kb on 1% agarose gel (data not shown). The analyses of the obtained 16S rRNA sequences indicated that the strains KG 2.2 and KG 2.5 belong to *Bacillus subtilis*, KG 2.3 is *B. amyloliquefaciens*with 99.9% DNA identity (Figure 3).

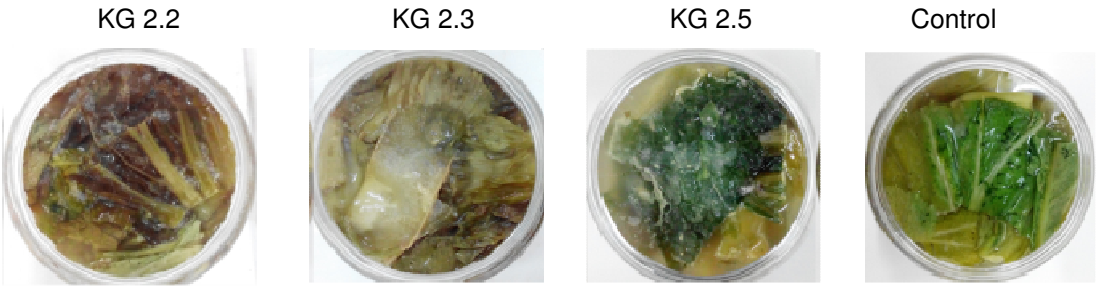


Figure 2. The white biofilm layer in fermentation jar with the spoilage bacteria after 24h.

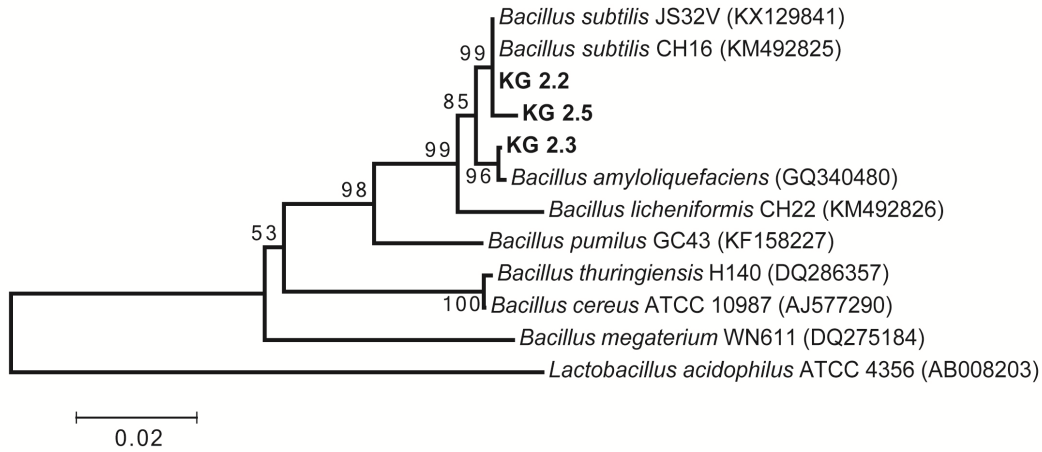


Figure 3. Phylogenetic analysis of the pickle-spoilage bacteria. Genetic distance is indicated by the scale bar and *Lactobacillus acidophilus* is used as root of the tree.

Recent reports indicated that fermented food-spoilage microbes are mainly yeasts and molds [3, 8]. These fungi possess various extracellular enzymes for digestion of complex substrates and can grow strongly in different fermentation conditions, especially their ability of organic acid tolerance [3]. However in this study, we have identified the first time some *Bacillus*strains with ability of fast growth in fermented pickles resulting in food spoilage. The overgrowth of these bacteria increased the pH and inhibited the fermentation process.

### *3.2. The yeast strainswith antibacterial activityisolated fromfermented pickles*

The yeast strainswere isolated from good fermented pickles and screened for antimicrobial activity against the spoilage bacteria. All yeast strains could grow in the cultivation conditions with the low pH. The antagonisticcapacity of these yeast against the spoilage bacteria was tested using agar well diffusion method. Our results indicated that three strains representing strong antibacterial activity (Table 2, Figure 3) with typical morphological characteristics for colonies and cells of yeasts (Table 3, Figure 4).

The yeast strain L36 is able tostrongly inhibitall three pickle-spoilage bacteria (Table 2). In morphology, this yeast strain has the smooth, deeply pink colonies and the cells in oval shape in a mixture with pseudohyphae (Table 3, Figure 4). This strain was selected for molecular taxonomy by sequencing the ITS region of rDNA.

Analysis of the rDNA ITS sequencesfor the strain L36 demonstrated that this yeast strain belongs to *Candida tropicalis* (Figure 5). The phylogenetic tree was generated with MEGA6 using Neighbor-joining method and 1,000 bootstrap replicates.

*Candida tropicalis*is used for biotransformation of polysaccharides into industrially important oligosaccharides such as xylitol, or for production of bioethanol [9]. However, little is known about this yeast for antimicrobial activity. This is first report about a *Candida tropicalis* strain with antagonistic activity against pickle-spoilage bacteria isolated from the traditional fermented vegetable products.

Table 2. Antibacterial activity of the yeast strains.

| Bacterial strains | Inhibition activity (mm) of the yeast strains | | |
|---|---|---|---|
| | L7 | L30 | L36 |
| **KG 2.2** | 4 | 7 | 13 |
| **KG 2.3** | 12 | 1 | 14 |
| **KG 2.5** | 12 | 12 | 13 |

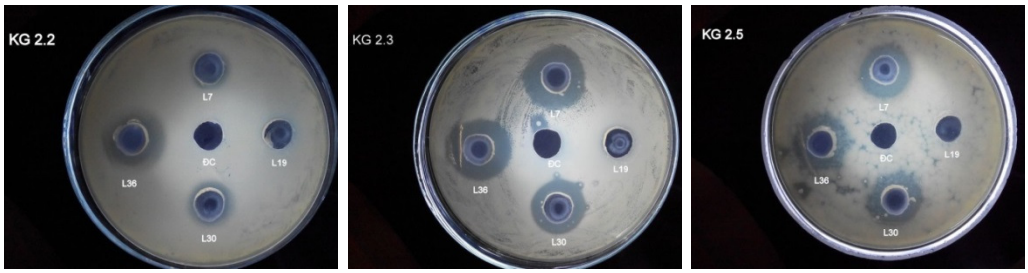


Figure 3. Antibacterial activity of the yeast strains against the pickle-spoilage bacteria.

Table 3. Morphological characteristics of the yeast strains.

| Morphology | Yeast strains | | |
|---|---|---|---|
| | L7 | L30 | L36 |
| **Colony** | Light pink, smooth, dry, circular, umbonate in center | Light pink, rough, dry, circular edge | Deeply pink, smooth, circular edge |
| **Cell** | Oval shape | Oval shape | Oval shape, pseudohyphae |

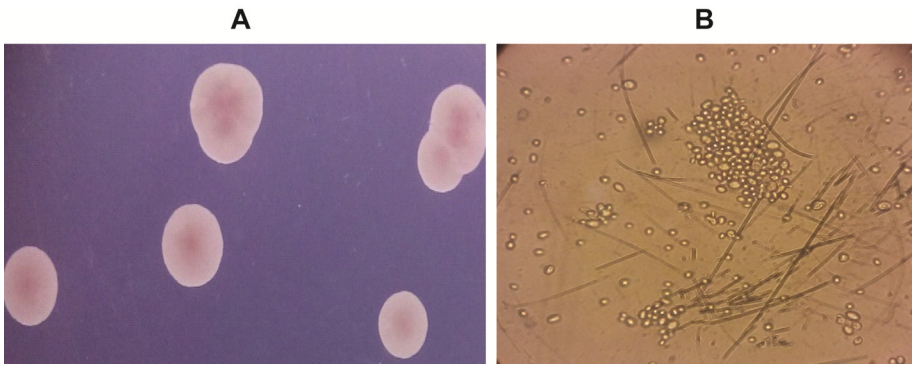


Figure 4. Morphology of the yeast strain L36.
(A) yeast colonies on Martin agar plate, (B) yeast cells under microscope.

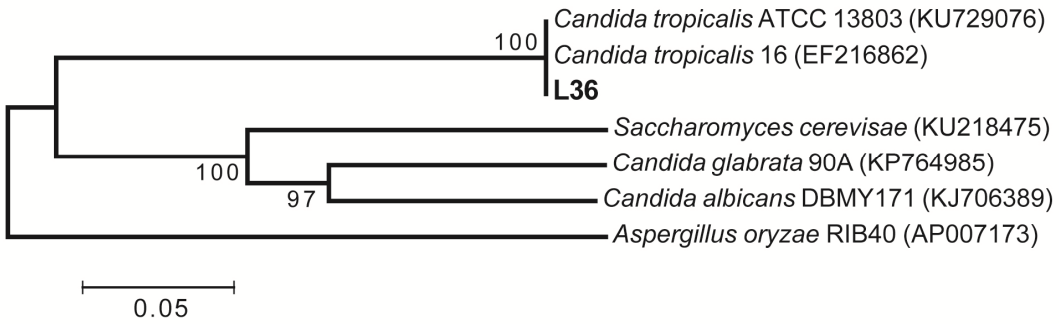


Figure 5. Phylogenetic analysis of the yeast strain L36.
Genetic distance is indicated by the scale bar and *Aspergillus oryzae* is used as root of the tree.

## 4. Conclusion

Three pickle-spoilage bacterialstrains were isolated and identified as *Bacillus* species including *Bacillus subtilis* (KG 2.2, KG 2.5) and *B. amyloliquefaciens* (KG 2.3). These strains were confirmed to cause pickle spoilage *in vitro*. One yeast strain (L36) having strong antimicrobial activity againstthe above spoilage bacteria was classified as *Candida tropicalis* based on morphological characteristics and the analysis of the rDNA ITS sequence. This yeast strain is a promising candidate as supplement for biopreservation of traditional fermented vegetable products.

## References

[1] Nyambane B, Thari WM, Wangoh J, Njage PMK, Lactic acid bacteria and yeasts involved in the fermentation of amabere amaruranu, a Kenyan fermented milk, Food Science and Nutrition 2(6) (2014) 692.

[2] Franco W, Pérez-Díaz IM, Johanningsmeier SD, McFeeter RF, Characteristics of spoilage-associated secondary cucumber fermentation, Applied and Enviromental Microbiology 78(4) (2012) 1273.

[3] Khan SH, Muhamad F, Idrees M, Shafique M, Farooqi IHMH, Some studies on spoilage fungi of pickles, Jounal of Agriculture and Social science1(1) (2005) 14.

[4] Rawat S, Food Spoilage: Microorganisms and their prevention, Asian Journal of Plant Science and Research 5(4) (2015) 47.

[5] Etchells JL, Costilow RN, Bell TA, Identification of yeasts from commercial cucumber fermentations in northern brining areas, Farlowia4 (1952) 249.

[6] Mai Thi Dam Linh, Nguyen Thi Giang, Nguyen Thi Tuyet, Kieu Huu Anh, Study on characteristics of lactic acid bacteria isolated in Hanoi. VNU Journal of Science: Natural Sciences and Technology 24 (2008) 221.

[7] Nguyen Thi Khuyen, Vo Thi Hanh, Pham Thi Hien, Mai Thi Dam Linh, Tran Duc Long, Tran Thi Thuy Anh, Trinh Tat Cuong, Tran Van Tuan (2015). A modified method of fungal DNA extraction for molecular diagnosis to discriminate between Aspergillus oryzae and Aspergillus flavus. VNU Journal of Science: Natural Sciences and Technology31 (4S) (2015) 167.

[8] Loureiro V, Malfeito-Ferreira M, Spoilage yeasts in the wine industry, International Journal of Food Microbiology 86(1)( 2003) 23.

[9] Satyanarayana T, Kunze G, Yeast Biotechnology: Diversity and Applications, Springer Science & Business Media, 2009.

# Phân lập và định danh chủng nấm men mới thuộc loài *Candida tropicalis* có khả năng kháng lại vi khuẩn gây hỏng sản phẩm dưa muối

Nguyễn Thị Thanh Mai[1], Mai Thị Đàm Linh[2], Nguyễn Phương Thu[2], Trần Văn Tuấn[2]

[1]*Trung tâm Sinh học Thực nghiệm, Viện Ứng dụng Công nghệ, Bộ KH&CN, Lê Thánh Tông, Hoàn Kiếm, Hà Nội, Việt Nam*
[2]*Bộ môn Vi sinh vật học, Khoa Sinh học, Trường Đại học Khoa học Tự nhiên, ĐHQGHN, 334 Nguyễn Trãi, Thanh Xuân, Hà Nội, Việt Nam*

**Tóm tắt:** Các loại rau lên men (dưa muối) truyền thống của Việt Nam lên men tự nhiên với sự có mặt của vi khuẩn lactic và các nhóm vi sinh vật khác. Vi khuẩn axit lactic đóng vai trò quan trọng trong quá trình lên men rau củ, trong khi một số nấm men được biết đến với vai trò hình thành hương vị cho sản phẩm. Tuy nhiên, các sản phẩm lên men đôi khi bị hư hỏng do nhiễm phải các vi sinh vật gây hỏng trong suốt quá trình lên men. Trong nghiên cứu này, chúng tôi phân lập ba chủng vi khuẩn (KG 2.2, KG 2.3, KG 2.5) và đã chứng minh được sự tăng trưởng của các vi khuẩn này đã gây hỏng sản phẩm lên men với sự hình thành của các lớp váng. Dựa trên các đặc điểm về hình thái và sinh hóa, các chủng vi khuẩn thuộc chi *Bacillus*. Kết quả giải trình tự và phân tích gen 16S rRNA cho thấy chủng KG 2.2 và KG 2.5 thuộc loài*Bacillus subtilis*, KG 2.3 là *B. amyloliquefaciens*. Đồng thời, chúng tôi cũng tiến hành phân lập và thu được ba chủng nấm men từ các mẫu dưa muối chất lượng cao. Những chủng nấm men này có thể ức chế mạnh sự phát triển của các chủng vi khuẩn gây hỏng dưa muối. Một trong những chủng có hoạt tính đối kháng mạnh nhất (chủng L36) đã được xác định là thuộc loài *Candida tropicalis* dựa trên trình tự ITS của rDNA. Đây là báo cáo đầu tiên về một chủng nấm men có hoạt tính kháng khuẩn chống lại các vi khuẩn gây hỏng thực phẩm muối chua. Chủng nấm này có thể được ứng dụng vào trong thực tiễn để bảo quản, cải thiện chất lượng của các sản phẩm rau quả lên men.

*Từ khóa:* Sản phẩm lên men, vi khuẩn gây hỏng, nấm men *Candida tropicalis*, hoạt tính kháng khuẩn.