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Effects of the cultivation conditions on antimicrobial activity of *Lactiplantibacillus* sp. NCL33 isolated from Nem chua

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Abstract

There is an increasing demand from consumers for natural and minimally processed foods. Therefore, developing and producing effective natural antimicrobial substances for food preservation is of great interest. This work aimed to investigate the effect of growth conditions on the maximum activity of antibacterial substances production by *Lactiplantibacillus* sp. NCL33 from Nem chua, a traditional fermented meat. The results revealed that the antibacterial substances exhibited a broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria. The antibacterial activity of NCL33 was optimized with MRS broth supplemented with 20 g/L glucose. The suitable nitrogen source for antibacterial biosynthesis was yeast extract with a concentration of 25 g/L. Moreover, the inoculated temperature and initial pH significantly influenced the antimicrobial activity. The maximum antimicrobial activity of NCL33 was obtained at 30°C and pH 7.0.

Keywords: *Lactiplantibacillus sp., antimicrobial activity, cultivation conditions.*

1. INTRODUCTION

The production of fermented meat products is part of the gastronomic habits of human culture from all continents. Each fermented meat product can be considered a heritage and culinary identity. The meat fermentation process involves various changes in physical, biochemical, and microbial aspects of the meat product due to the activities of microorganisms. The flavor of fermented meat products is mainly attributed to the presence

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of lactic acid and low-molecular-weight odor-active compounds such as peptides, free amino acids, and aldehydes derived from meat protein breakdown [1]. The addition of starter cultures is essential to improve the quality and enhance the sensory characteristics of fermented meat products. Moreover, starter cultures may also include microorganisms capable of producing beneficial health-promoting compounds, exhibiting probiotic properties, or reducing the levels of amines and cholesterol in fermented meat products [2].

Probiotics are selected live microorganisms that, when administered in adequate amounts, provide health benefits to the host, mainly belonging to *Bifidobacterium* and *Lactobacillus*. Some species of the genera *Lactococcus*, *Enterococcus*, *Saccharomyces*, and *Propionibacterium* have also been reported to exhibit probiotic activity [3]. Several studies have demonstrated the effectiveness of supplementing probiotic starter cultures in producing fermented meat. Erkkila et al. (2001) examined the fermentation efficiency of three probiotic strains of *Lactobacillus rhamnosus* to produce dry sausages [4]. Todorov et al. (2013) investigated the safety of *Lactobacillus sakei*, *Lactobacillus plantarum*, and *Enterococcus faecium* strains isolated from Portuguese fermented meat products for application as starter cultures, bacteriocin production, or probiotics [5]. In Vietnam, Nguyen Hoai Huong et al. (2011) also selected lactic acid bacteria from traditional fermented sausages as probiotic starter cultures for Nem Chua production [6].

Starter cultures with antimicrobial properties are advantageous due to their potential competitive interactions with pathogenic bacteria in food. Nisin, the first lactic acid bacteriocin, is approved in over 40 countries and has been used as a food preservative for over 50 years. Several other antimicrobial substances produced by lactic acid bacteria have been reported in recent years [7]. Nevertheless, more research is needed to develop novel natural antimicrobial substances with a broad spectrum.

This study determined the broad-spectrum antibacterial characteristics of *Lactiplantibacillus* sp. strain NCL33 isolated from fermented sausage. Additionally, cultivation conditions aimed at enhancing the antibacterial activity of this bacterial strain were investigated. The results of this study provide additional information on the beneficial effects of lactic acid bacteria from Nem Chua and expand their applications as starter cultures and in controlling pathogenic microorganisms.

2. MATERIALS AND METHODS

2.1. Materials

The bacterial strains used in the study included *Lactiplantibacillus* sp. NCL33 isolated from Nem Chua collected in Thanh Hoa province, and the indicator bacteria *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Salmonella enterica* ATCC 13076, *Pseudomonas aeruginosa* ATCC 27853, and *Bacillus cereus* ATCC 14579. These strains were stored in 15% glycerol at -80°C at the Bioresource Research Center, Phenikaa University. Prior to the experiments, *Lactiplantibacillus* sp. NCL33 strain was incubated on

MRS medium (g/L: bacto peptone - 10, beef extract - 10, yeast extract - 5, glucose - 20, ammonium citrate - 2, sodium acetate - 5, magnesium sulfate - 0.1, manganese sulfate - 0.05, dipotassium phosphate - 2, and Tween 80 - 1 mL/L) at 30°C, while the indicator strains were cultured on LB medium (g/L: NaCl - 10, yeast extract - 5, peptone - 10, agar - 15) at 37°C.

2.2. Methods

2.2.1. Antimicrobial activity assay

The antibacterial activity of the NCL33 strain was determined using the agar diffusion method, as described by Yue et al. in 2013 [8]. The indicator bacteria were grown aerobically (160 rpm) at 37ºC overnight. Semi-solid LB medium supplemented with 1.0 % agar was mixed with approximately 10^5 CFU/mL of the indicator strain and allowed to solidify. The 24-hour NCL33 cultures were centrifuged at 10,000 rpm for 5 minutes. The supernatant was adjusted to pH 6.5 using 3N NaOH. The NCL33 strain was cultured on MRS medium at pH 7.0, 37°C, 160 rpm for 48 hours, then centrifuged at 8,000 rpm for 15 minutes to remove cells. The pH was adjusted to 6.5 with 3N NaOH. Wells of 5 mm diameter were perforated using a sterile border, and 50 µL of the cell-free supernatant was placed into each well. The agar plates were kept at 4°C for 4 hours, followed by incubation at 37°C, and examined after 24 h. The antibacterial activity was determined by measuring the diameter of the inhibition zone on the agar plates. The experiment was repeated three times.

2.2.2. The effect of culture conditions on the production of Lactiplantibacillus sp. NCL33 antimicrobial substances

To investigate the effects of different conditions on the antibacterial activity of the NCL33 strain, five factors, including carbon source, nitrogen source, initial pH, and temperature, were selected for examination. *Pseudomonas aeruginosa* ATCC 27853 was used as the indicator bacterium. The NCL33 strain was cultured in 250 mL flasks containing 75 mL of MRS medium (g/L: bacto peptone - 10, beef extract - 10, yeast extract - 5, glucose - 20, Tween 80 - 1 mL/L, ammonium citrate - 2, sodium acetate - 5, magnesium sulfate - 0.1, manganese sulfate - 0.05, dipotassium phosphate - 2). The cultivation parameters, including the initial inoculum ratio of 2% (v/v), agitation of 160 rpm, and culture time of 48 hours, were kept constant in all experiments. The experiments were repeated three times.

Carbon source: The NCL33 strain was cultured in MRS medium (without glucose) containing different sugar sources, including lactose, glucose, maltose, and sucrose (20 g/L). The optimal carbon source was selected and studied at 5, 10, 20, 30, and 40 g/L.

Nitrogen source: The NCL33 strain was cultured in a broth medium (following MRS formulation but without a nitrogen source), and different nitrogen sources were then added to the broth medium. These were either (A) 10 g/L bacto peptone plus 10 g/L beef extract and 5 g/L yeast extract, or (B) 25 g/L bacto peptone, or (C) 25 g/L beef extract, or (D) 25 g/L yeast extract. The optimal nitrogen source was then selected and studied at 10, 20, 25, 30, and 40 g/L.

Initial pH: The effect of initial pH on the antibacterial activity of the NCL33 strain was determined by adjusting the initial pH of the MRS broth to 4.0, 5.0, 6.0, 7.0, 8.0, to 9.0.

Culture temperature: The optimal temperature was examined by conducting growth experiments at 25, 30, 37, 42°C.

2.2.3. Statistical analysis

The data were analyzed using Excel software. All assays were carried out in triplicate, and the results were expressed as mean values ± standard deviation. Significant divergences among mean values were determined using the ANOVA statistical method. A p-value of < 0.05 was considered significant.

3. RESULTS AND DISCUSSION

3.1. Antibacterial activity against five pathogenic bacterial strains by *Lactiplantibacillus sp. NCL33*

The antibacterial activity of the NCL33 strain was determined using the agar diffusion method, and the results are shown in Figure 1. The NCL33 strain exhibited the ability to simultaneously inhibit five pathogenic bacterial strains, including three Gram-negative strains (*Escherichia coli*, *Salmonella enterica*, *Pseudomonas aeruginosa*) and two Gram-positive strains (*Staphylococcus aureus*, *Bacillus cereus*). The diameter of the antibacterial zone of the NCL33 strain against the indicator strains ranged from 11 to 14.5 mm, with the highest inhibition observed against *Pseudomonas aeruginosa*.

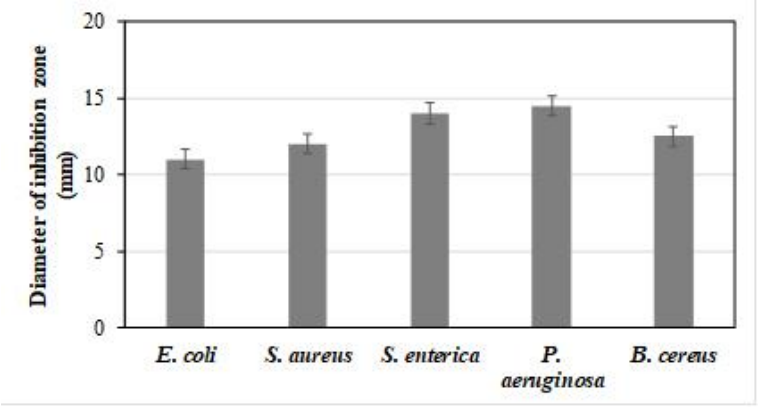

***Figure. 1**. Antibacterial activity against five pathogenic bacterial strains by NCL33 strain*

The antibacterial mechanism of lactic acid bacteria (LAB) is known to be multifactorial. Antibacterial compounds from LAB may include lactic acid, which primarily contributes to reducing pH in the product. Besides lactic acid, the presence of other metabolites such as diacetyl, alcohol, bacteriocins, and other organic acids may also be related to the antibacterial activity of LAB. Many LAB strains isolated from fermented meat samples have been found to possess bacteriocin production capabilities [9]. Phan et al. (2005) isolated *Lactobacillus plantarum* H1.40 strain, which exhibited good activity against strains of *S. aureus*, *B. cereus*, *Listeria monocytogenes*, *S. typhimurium*, and *E. coli* [10].

From hundreds of LAB strains isolated from 46 fermented foods, Elegado et al. (2003) discovered nine strains with bacteriocin production abilities [11]. The cultivation method can also affect the bacteriocin production ability of LAB. Some LAB strains only produce bacteriocins when co-cultured with specific Gram-positive strains or supplemented with heat-killed cells of certain inducing strains, such as *L. plantarum* NC8 and *L. plantarum* C11 [12]. In this study, the lactic acid bacteria strain NCL33 exhibited broad-spectrum antibacterial activity and great potential for application as a natural food preservative agent.

3.2. Effects of cultivation conditions on the antibacterial activity of *Lactiplantibacillus* sp. NCL33

The results of the effects of four selective growth factors (carbon source, nitrogen source, initial pH, temperature) on the antibacterial activity of the NCL33 strain are shown in Figure 2.

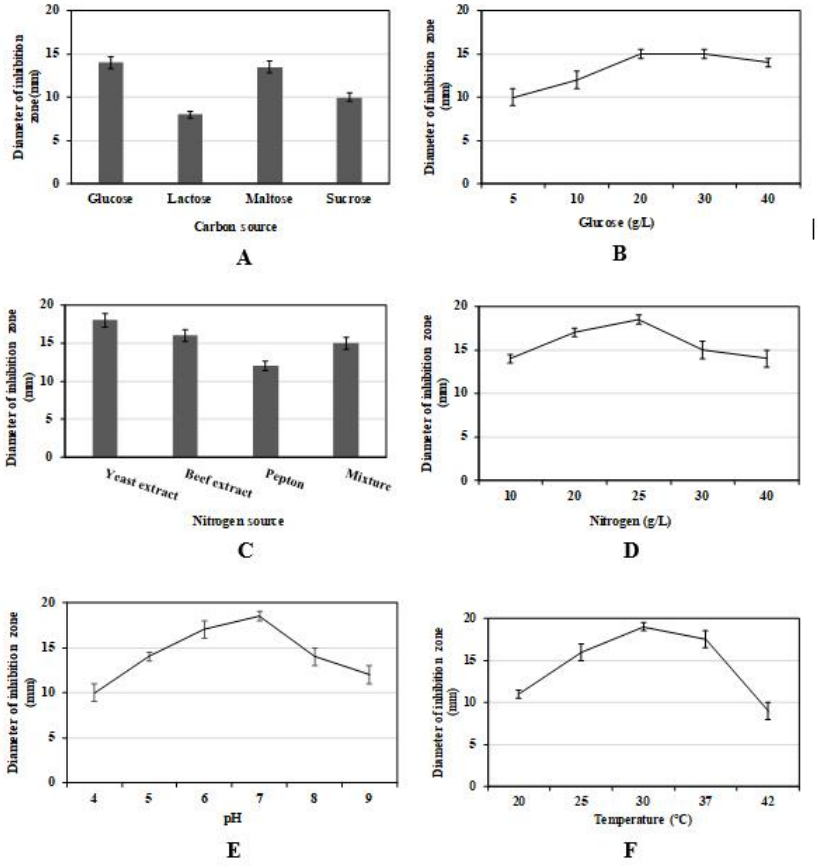

***Figure 2**. Effect of carbon source (A, B), nitrogen source (C, D), initial pH (E), temperature (F) on antimicrobial activity*

Carbon source is known to be the primary carbon and energy source for bacteria, making it an essential component in the culture medium for the growth and typical functional properties of LAB. However, the optimal carbon source depends on each different LAB strain. *L. fermentum* Ogi E1 preferred maltose over starch, glucose, or melibiose [13]. The suitable carbon sources for the growth and bacteriocin production of *L. plantarum* B21 were

glucose and maltose [14]. In this study, the *Lactiplantibacillus* sp. NCL33 strain exhibited the highest antibacterial activity in MRS medium containing glucose, with an antibacterial activity zone diameter of 14 mm, followed by maltose and lactose (Figure 2A). When varying the glucose concentration from 5, 10, 20, 30 to 40 g/L, the antibacterial activity of the NCL33 strain gradually increased from a concentration of 5 - 30 g/L. However, the antibacterial capability of the NCL33 strain decreased when the glucose concentration reached 40 g/L (Figure 2B).

The effect of nitrogen source on the antibacterial efficacy of the NCL33 strain is indicated in Figure 2C. Some nitrogen sources commonly used for LAB cultivation include beef extract, yeast extract, peptone, tryptone, and milk protein hydrolysate. Among them, beef extract, yeast extract, and peptone are the most frequently used for LAB cultivation. However, substituting a specific nitrogen source can significantly impact LAB's growth and metabolism processes. For example, replacing half of the yeast extract with beef or malt extract has been shown to reduce the growth and bacteriocin production of *L. sakei* CCUG 42678 [15]. Substituting tryptone with peptone or soytone enhances the growth and bacteriocin production of *L. sakei* G20 and R04 [16] for the *Lactiplantibacillus* sp. NCL33 strain, using yeast extract as the nitrogen source, resulted in the highest antibacterial activity compared to other nitrogen sources such as beef extract, peptone, or a mixture of the three. The most suitable yeast extract concentration was determined to be 25 g/L, and increasing or decreasing the yeast extract concentration both reduced the antibacterial activity of the NCL33 strain (Figure 2D).

The effect of initial pH on the antibacterial activity of NCL33 strain is shown in Figure 2E. The antibacterial activity of NCL33 strain was increased when the initial pH of the medium was 7.0, with the largest diameter of the antibacterial zone reaching 18.5 mm. Conversely, acidic (low initial pH of 4.0) or alkaline (8.0, 9.0) conditions reduced the antibacterial activity of NCL33 strain. Similar findings were reported for the antibacterial activity of strains *L. plantarum* ST23LD [17], *L. plantarum* ST13BR [18], and *L. plantarum* bacST202Ch [19]. These LAB strains exhibited optimal antibacterial activity in a medium with an initial pH of 6.5.

The effect of temperature on the antibacterial activity of NCL33 strain was shown in Figure 2F. The results demonstrated that the largest diameter of the antibacterial zone, measuring 19 mm, was achieved at a cultivation temperature of 30°C. Yang et al. (2018) reported that the growth and production of bacteriocin in *Lactobacillus curvatus* Arla-10, *Enterococcus faecium* JFR-1, *L. paracasei* JFR-5, and *Streptococcus thermophilus* TSB-8 were highest when cultured in MRS medium at 37°C [20]. However, Mataragas et al. (2003) reported that the optimal temperature for the growth of *Leuconostoc mesenteroides* L124 and *L. curvatus* L442 was 30°C, while bacteriocin production occurred at 25°C [21]. In this study, the optimal temperature that enhanced the antibacterial activity of *Lactiplantibacillus* sp. NCL33 was 30°C. Generally, the optimal temperature for antibacterial activity in LAB varies depending on the strain and typically ranges from 30 to 37°C [22].

4. CONCLUSION

Lactiplantibacillus sp. NCL33 exhibited the best antibacterial activity in an MRS medium supplemented with 20 g/L glucose as the carbon source and 25 g/L yeast extract as the nitrogen source. The suitable culture temperature and initial pH for the antibacterial activity of NCL33 were 30°C and pH 7.0, respectively. Under optimal cultivation conditions, the diameter of the antimicrobial zone reached a maximum of 19 mm, representing a 36% increase compared to the initial culture conditions.

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REFERENCES

[1]. E. Claeys, S. De Smet, A. Balcaen, K. Raes, and D. Demeyer, "Quantification of fresh meat peptides by SDS-PAGE in relation to ageing time and taste intensity," *Meat Science,* vol. 67, pp. 281-288, 2004.

[2]. F. Leroy, J. Verluyten, and L. de Vuyst, "Functional meat starter cultures for improved sausages fermentation," *International Journal Food Microbiology,* vol. 106, pp. 270-285, 2006.

[3]. Y. Zhang, J. Z. Liu, J. S. Huang, and Z. W. Mao, "Genome shuffling of Propionibacterium shermanii for improving vitamin B12 production and comparative proteome analysis," *Journal of Biotechnology*, vol. 148, pp. 139-143, 2010.

[4]. S. Erkkila, E. Peteja, S. Eerola, L. Lilleberg, T. Mattila-Sandholm, and M. L. Suihko, "Flavour profiles of dry sausages fermented by selected novel meat starter cultures," *Meat Science,* vol. 58, pp. 111-116, 2001.

[5]. S. D. Todorov, B. D. G. M. Franco, and I. J. Wiid, "In vitro study of beneficial properties and safety of lactic acid bacteria isolated from Portuguese fermented meat products," *Beneficial Microbes*, vol. 5, pp. 351-366, 2013.

[6]. N. H. Huong, D. T. Vy, T. L. Chau, and D. K. Nhu, "Selective isolation of lactic acid bacteria from traditional fermented meat (Nem chua) as starter strains of probiotic fermented meat (Nem chua)," Proceedings of the Conference on Environmental Science and Biotechnology, pp. 149-157, 2011.

[7]. J. Miao, M. Xu, H. Guo, L. He, X. Gao, C. DiMarco-Crook, H. Xiao, and Y. Cao, "Optimization of culture conditions for the production of antimicrobial substances by probiotic *Lactobacillus paracasei* subsp. Tolerans FX-6," *Journal of Functional Foods*, vol 18, pp. 244-253, 2015.

[8]. T. L. Yue, J. J. Pei, and Y. H. Yuan, "Purification and characterization of anti - Alicyclobacillus bacteriocin produced by *Lactobacillus rhamnosus*," *Journal of Food Protection*, vol. 76, pp. 1575-1581, 2013.

[9]. S. Ammor, G. Tauveron, E. Dufour, and I. Chevallier, "Antibacterial activity of lactic acid bacteria against spoilage and pathogenic bacteria isolated from the same meat small-scale facility. Screening and characterization of the antibacterial compounds," *Food Control,* vol. 17, pp. 454-461, 2005.

[10]. T. T. Phan, T. H. Do, L. H. Tran, and D. H. Hoang, "Isolation and characterization of *Lactobacillus plantarum* H1.40 from Vietnamese traditional fermented meat (Nem

chua)," In: Proceedings of Regional Symposium on Chemical Engineering, 30 November-2 December 2005, Hanoi, (in Vietnamese).

[11]. F. B. Elegado, A. C. L. Opina, C. G. B. Banaay, I. F. and Dalmacio, "Purification and characterization of novel bacteriocins from lactic acid bacteria isolated from Philippine fermented rice-shrimp or rice-fish mixtures," *The Philippine Agricultural Scientist*, vol. 86, pp. 65-74, 2003.

[12]. A. Maldonado, R. Jimenez-Diaz, J. L. and Ruiz-Barba, "Induction of plantaricin production in *Lactobacillus plantarum* NC8 after co-culture with specific Gram-positive bacteria is mediated by an auto-induction mechanism," *Journal of Bacteriology*, vol. 186, pp. 1556-1564, 2004.

[13]. M. Calderon, G. Loiseau, J. P. Guyot, "Nutritional requirements and simplified cultivation medium to study growth and energetics of a sourdough lactic acid bacterium *Lactobacillus fermentum* Ogi E1 during heterolactic fermentation of starch," *Journal Applied Microbiology*, vol. 90, pp. 508-516, 2001.

[14]. E. Parlindungan, C. Dekiwadia, O. A. H. Jones, "Factors that influence growth and bacteriocin production in *Lactiplantibacillus plantarum* B21", *Process Biochemistry*, vol 107, pp. 18-26, 2021.

[15]. I. M. Aasen, T. Moretro, T. Katla, L. Axelsson, I. Storro, "Influence of complex nutrients, temperature and pH on bacteriocin production by *Lactobacillus sakei* CCUG 42687," *Applied Microbiology Biotechnology*, vol. 53, pp. 159-166, 2000.

[16]. T. Lechiancole, A. Ricciardi, E. Parente, "Optimization of media and fermentation conditions for the growth of *Lactobacillus sakei*," *Annals Microbiology*, vol. 52, pp. 257-274, 2002.

[17]. S. D. Todorov, L. M. Dicks, "Effect of medium components on bacteriocin production by *Lactobacillus plantarum* strains ST23LD and ST341LD, isolated from spoiled olive brine," Microbiology Research, vol. 161, pp. 102-108, 2006.

[18]. S. D. Todorov, C. A. Van Reenen, L. M. T. Dicks, "Optimization of bacteriocin production by *Lactobacillus plantarum* ST13BR, a strain isolated from barley beer," *The Journal of General and Applied Microbiology*, vol. 50, pp. 149-57, 2004.

[19]. S. D. Todorov, P. Hob, M. Vaz-Velho, and L. M. T. Dicks, "Characterization of bacteriocins produced by two strains of *Lactobacillus plantarum* isolated from Beloura and Chouriço, traditional pork products from Portugal," *Meat Sciences*, vol. 84, pp. 334-343, 2010.

[20]. E. Yang, L. Fan, J. Yan, Y. Jiang, C. Doucette, S. Fillmore, and B. Walker, "Influence of culture media, pH and temperature on growth and bacteriocin production of bacteriocinogenic lactic acid bacteria," *AMB Express*, vol. 8, 2018.

[21]. M. Mataragas, J. Metaxopoulos, M. Galiotou, and E. H. Drosinos, "Influence of pH and temperature on growth and bacteriocin production by *Leuconostoc mesenteroides* L124 and *Lactobacillus curvatus* L442," *Meat Science*, vol. 64, pp. 265-271, 2003.

[22]. F. Ibrahim, S. B. Zafar, A. Aman, S. A. Ul Qader, and A. Ansari, "Improvement of *Lactobacillus plantarum* for the enhanced production of bacteriocin like inhibitory substance using combinatorial approach," *Biocatalysis Agricultural Biotechnology*, vol. 22, 101386, 2019.

Lựa chọn điều kiện nuôi cấy thích hợp nhằm tăng cường khả năng kháng khuẩn của chủng *Lactiplantibacillus sp.* NCL33 phân lập từ nem chua

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Tóm tắt

Để đáp ứng nhu cầu ngày càng tăng của người tiêu dùng đối với các sản phẩm tự nhiên và thực phẩm chế biến tối thiểu, các nhà khoa học đã tập trung nghiên cứu và phát triển các hợp chất kháng khuẩn tự nhiên để bảo quản thực phẩm. Mục đích của nghiên cứu này là khảo sát ảnh hưởng của các điều kiện sinh trưởng lên khả năng kháng khuẩn của chủng *Lactiplantibacillus* sp. NCL33 phân lập từ nem chua. Chủng NCL33 có phổ kháng rộng với nhiều chủng vi khuẩn Gram âm và Gram dương. Chủng NCL33 kháng khuẩn tốt nhất trong môi trường MRS bổ sung glucose nồng độ 20 g/L. Trong khi đó, nguồn nitơ thích hợp là cao nấm men với nồng độ 25 g/L. Ngoài ra, nhiệt độ và pH ban đầu của môi trường cũng được xác định là có ảnh hưởng đến hiệu quả kháng khuẩn của chủng NCL33. Chủng NCL33 kháng khuẩn tốt nhất khi nhiệt độ nuôi cấy là 30°C và pH ban đầu là 7,0.

Từ khóa: *Lactiplantibacillus sp., hoạt tính kháng khuẩn, điều kiện nuôi cấy.*