ISBN 978-979-19546-1-7

PROCEEDING

The 3rd International Conference of Indonesian Society for Lactic Acid Bacteria (3rd IC-ISLAB)

Better Life with Lactic Acid Bacteria
Exploring Novel Functions of Lactic Acid Bacteria

Editors

Prof. Dr. Ir. Endang S. Rahayu
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Auditorium
Faculty of Agricultural Technology
Gadjah Mada University
Yogyakarta

March, 2011

In collaboration with

Indonesian Society for Microbiology (PERMI)

Faculty of Agricultural Technology, Gadjah Mada University, Yogyakarta, INDONESIA

Organized by

Indonesian Society for Lactic Acid Bacteria

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PROCEEDING

The 3rd International Conference of Indonesian Society for Lactic Acid Bacteria (3rd IC-ISLAB) : "Better Life with Lactic Acid Bacteria; Exploring Novel Functions of Lactic Acid Bacteria"

Auditorium Faculty of Agricultural Technology,
Gadjah Mada University
Yogyakarta, 21-22 January, 2011

ISBN: 978-979-19546-1-7

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Preface
Chairman of Organizing Committee

It is well known that several researches in area of lactic acid bacteria (LAB) have been conducted in isolation and characterization of various indigenous microorganisms linked to food fermentation which provide probiotic and prebiotic ability as well as additional synergic suppression and inhibition for spoilage and pathogenic microorganism. Many species and strain of LAB's have been suggested to provide beneficial effects on the gastrointestinal tract of human and animal. However, the most challenging effort is not only introduction the potential of indigenous microorganism but how to apply the research output into community. Therefore, the selection of the topic conference entitled *Better Life with Lactic Acid Bacteria: Exploring Novel Functions of Lactic Acid Bacteria* is one of the efforts to solve those challenging. Emphasizing of the research dissemination into society through strengthen the research networking among scientists, government, business society, and community is the main objective in this 3rd International Conference of Indonesian Society for Lactic Acid Bacteria (IC-ISLAB).

Since it was established on March 12, 2003 in the Faculty of Agricultural Technology Gadjah Mada University, the ISLAB has conducted two international conferences in 2005 and 2009. Two years ago (January 16 – 17, 2009), the 2nd IC-ISLAB was held in the Faculty of Agricultural Technology, and again this year, the 3rd international conference has also organized in this faculty. Learning from the previous conference, the increasing of participant from researchers, food and pharmaceutical manufactures, pediatricians, culture collection curators, government institutions, and students has been achieved for the number and country of origin. According to the participant list, the organizing committee has received 28 papers of oral presentation in the technical session, 51 papers of poster presentation, and 16 papers of presentation delivered by 4 Indonesian invited speaker and 12 overseas speakers (Austria, India, Japan, Korea, Malaysia, Mongolia, Pakistan, and Singapore). The scientific meeting will be arranged in 2 plenary and 6 technical sessions as well as a poster session during two days conferences. Enhancing the networking strengthen among the participant would also be built from the social gathering since breakfast prior to opening conference up to the conference dinner.

Ending this preface, on the behalf of the organizing committee I would like to express my gratitude to Faculty of Agricultural Technology Gadjah Mada University, member of Indonesian Society for Lactic Acid Bacteria, Indonesian Microbiological Society (PERMI), and several sponsored institution from PT. Yakult Indonesia Persada, PT. Nestle Indonesia, PT. Dipa Puspa Labsains, PT. Yummy Food Utama, and Food Review Indonesia. The last but not the least are distinguish speakers and participants for their tremendous effort and time spent in this conference, without all of you the conference would not be held. In the last I wish you the successful scientific meeting and hopefully come to further collaboration for your research activities.

Yogyakarta, March 2011
Organizing Committee
Chairman,

Dr.nat.techn. Francis M.C. Sigit Setyabudi, STP, MP

Proceeding
The 3rd International Conference of Indonesian Society for Lactic Acid Bacteria (3rd IC-ISLAB) : Better Life with Lactic Acid Bacteria: Exploring Novel Functions of Lactic Acid Bacteria.

DETECTION OF BACTERIOPHAGE INFECTED-CELL OF *LACTOCOCCUS LACTIS* SSP. *LACTIS* C2 USING ACOUSTIC EMISSION TECHNIQUE

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ABSTRACT

The objective of this study was to distinguish two lactococcal bacteriophage sk1 and ml3 by acoustic emission technique. In this technique, the signal was emitted from growth medium where L. lactis ssp. lactis C2 was infected with phages sk1 or ml3. The signal was monitored using contact piezoelectric sensors attached to the sides of the growth chambers. The two sensors (5 to 50 kHz range) had individual characteristic and internal amplification mechanisms that were calibrated and adjusted to minimize background noise. Sound intensity from the growth chambers was measured in attojoules (aJ = 10^{-18} Joules) and plotted as the energy rate-per-detected acoustic wave. Acoustic peaks considered significant and beyond internal or external generated noise were those having greater than ±3 times the sigma value of the general variation in acoustic intensity over the entire data set of each test. The acoustic data showed that energy rate from control tests in which L. lactis ssp. lactis C2 was grown without phage sk1 or phage ml3 infections contained no acoustic peaks with intensities that exceeded the ±3 sigma standard. On the contrary, multiple acoustic peaks with intensities that exceeded ±3 sigma were observed when phage sk1 or ml3 infected L. lactis ssp. lactis C2 culture. A significant difference between timing acoustic peaks of phage sk1 and ml3 were also observed. The first peaks for phage sk1 appeared at 33.2±4.4 min, whereas the first peak for phage ml3 appeared 40 min. Thus, these two phages could be distinguished by acoustic emission monitoring during phage infection of the bacteria. This new method can be performed in real time therefore allow fast and early detection of phage infected bacteria.

Keywords: acoustic emission, Lactococcus lactis, phage, piezoelectric sensors

INTRODUCTION

Bacteriophage infection in dairy fermentation constitutes a serious problem worldwide. In many of these applications, infection of the cultures by virulent bacteriophages is a problem leading to either complete loss of the fermentation or an altered flavor (4). The significance of bacteriophage infection of Lactic Acid Bacteria (LAB), most notably of members of the genus *Lactococcus*, is well recognized by industrial organizations worldwide. Their negative impact on several dairy fermentation processes was established and to the present day phages represent one of the most persistent problems in commercial practice (30). Several strategies have been employed to prevent phage infections such as to employ pure cultures and phage-resistant starter strains (4, 18). Detailed plans for rotational changes of phage resistant starter cultures have been worked out (26) to suppress the propagation of phage mutants that have overcome the resistance mechanism of the bacterial strains. The dairy industry has traditionally employed several physical and biological methods to deal with phage related problems, ranging from the implementation of aseptic processing conditions to the utilization of strain rotation regimes (20, 21). Although these strategies have had success in preventing

ISBN : 978-979-19546-1-7

phage infections, they cannot completely prevent bacteriophage attack and they may limit the number and diversity of dairy starter cultures available for production which may in turn limit the diversity of cheese flavours (18). Some work has also been carried out to develop methods that would detect phage infection at a sufficiently early stage where an operator might interfere with the process. A method based on the PCR technique for detection of infection by the three common phage species in dairy plants, the 936, c2 and P335 species was developed (19). A problem with PCR based analysis, however, is that it takes hours to complete the analysis and with the fast replication cycle of most bacteriophages, the fermentation batch will be destroyed before the result of the analysis is ready.

In this study, we describe a new method for detection of phage infection in *Lactococcus lactis*.. The method is based on acoustic detection, the method allows fast and early detection of phage-infected bacteria, independently of which phage has infected the culture. The method can be performed in real time and therefore increases the chance of successful intervention in the fermentation process. Acoustic emission (AE) measurements have been applied to measure events as diverse as micro-cracking in metals, earthquake tremors, chemical reactions and micro-bubble processes (2, 3, 24). Although AE studies of eukaryotic, prokaryotic or viral activity have not been published, it was nevertheless hypothesized that weak AE signals could be present because of physical and chemical processes that are known to occur during cellular activity, quorum sensing, metabolism, cell division, and cell death (1,2 3, 5, 9-12).

MATERIALS AND METHODS

Culture and Bacteriophage.

Lactococcus lactis ssp. *lactis* C2, bacteriophages sk1and ml3 were obtained from the University of Kentucky culture Library. Bacterial strains were maintained as frozen stocks at -80°C in M17 broth supplemented with 20% of glycerol, and routinely reactivated overnight at 26°C in M17 broth. Phage stocks were prepared in M17 broth, added of 10 mM $CaCl_2$ (M17-Ca) and then stored at -80°C.

Medium Preparation

M17 medium was purchased from Fischer laboratories and all other chemicals were purchased from Aldrich Chemical. Medium was prepared by adding 100 ml of 1M $CaCl_2.6H_2O$, 5 g of lactose to 1 L of M17 broth. The pH was adjusted to 6.9. All ingredients, except the lactose, were mixed together (separated into the appropriate aliquots) and sterilized at 121°C for 15 min. Lactose solution was sterilized separately for the same temperature and time. After sterilization, lactose solution was added to each aliquot. Four hundred (400) ml of sterilized medium was used to test the phage using acoustic monitoring device.

Bacteriophages Propagation

Twenty five (25) ml M17 medium was inoculated with 1 ml of *L. lactis* ssp. *lactis* C2 culture (1 x 10^9CFU/ml) and allowed to incubate for one hour in a 26°C water bath. At one hour, phage was inoculated at 250 µl (1 x 10^7pfu/ml) into the growth tube. $CaCl_2$ was added to the mixture at 250 µl of 0.1 M $CaCl_2$ solution. The mixture incubated in a 26 °C water bath until clearing or lysis of the culture was observed (approximately 4 h). The lysed culture was filtered through 0.45µ sterilized filter into a sterile bottle to remove cellular debris and phage resistant bacteria (from the phage). Phage (1 x

10^7pfu/ml) were stored in M17 broth in 1 ml aliquots and frozen at −80°C.

Medium Preparation for Enumeration of Bacteriophage

Bacteriophages were enumerated using a top/bottom agar assay Petri plate procedure as described by Hicks *et al.* (13).

Enumeration of Bacteriophage

C2 culture was propagated for 16 h at 26°C. Serial dilutions of bacteriophage were made. Top agar was steamed for 20 min and tempered to 45° C. C2 culture at 100 µl (1 x 10^9CFU/ml) and 100 µl of 0.1 M $CaCl_2$ was added to 2.5 ml top agar. The appropriate dilution of phage was added, vortexed and allowed to stand for 10 s. The mixture was then overlaid on the pre-prepared bottom agar. Plates were incubated at 26° C for 6 h uninverted. Plaques were counted after 6 h of incubation.

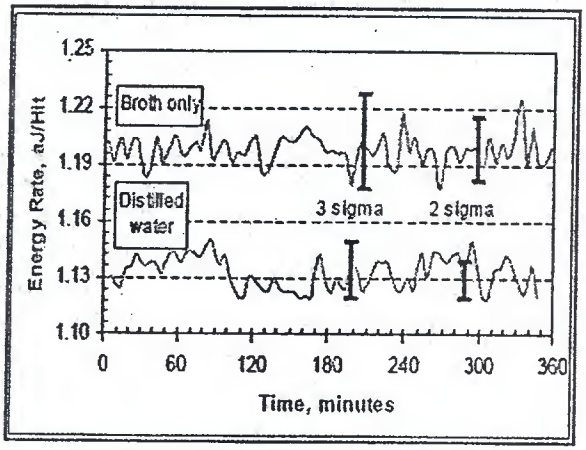
Acoustic monitoring device

A chamber having two identical temperature controlled compartments were sound insulated and vibration isolated to minimize interference from acoustic sources external to them. The AE sensors and software used were manufactured by Physical Acoustics Corporation (PAC, Princeton Junction, NJ).

The sensors (model number R6) had a sensitivity between 5 – 50 kHz. Acoustic signals were pre-amplified and then analyzed using PAC AEWin and AEPost software. A growth cell containing the sterilized medium was place in each chamber (26 °C) with two sensors attached to the cell. An additional guard sensor was attached to the cabinet to monitor acoustic noise from sources external to the growth cells. A thin layer of silicon grease was applied to the face of each sensor to enhance the acoustic signal transfer. The compartments were closed and background AE data were collected to establish threshold levels for each sensor. These levels were used as an input parameter to the software; if acoustic wave intensities above the sensor's threshold were detected the software enabled storage. *L. lactis* ssp. *lactis* C2 was used as the host to inoculate (1% or 4 ml of culture containing 10^9CFU/ml) the medium. The medium was stirred for 1 min prior to data collection (up to 8 h). Bacteriophages ml3 and sk1 were added to the medium at an infection level of 10^5pfu/ml (final concentration in growth cell) approx. 90 min after inoculation of the host.

Monitoring of M17 and Water

As a control both M17 medium and water (Figure 1) were monitored to determine if the noise level produce by these substances would exceed the 3σ level. Some noise signals exceeded 2σ, but none exceeded 3σ, thus 3σ was chosen as the point where AE could be attributed only to the treatment.



Note that No Data Exceeded the 3σ Level.

Monitoring of cell growth

A sterile pipette and collection tube was used to collect a sample (3 ml) at 30 min intervals. AE measurements were stopped, the cell was stirred for 1 min, the sample was collected, and the AE measurements were resumed when sampling was complete. The OD of the sample was determined at 600 nm, using a UNICO 1000 spectrophotometer, to estimate cell growth.

Statistical analysis

Analysis of AE was conducted using statistical software, where only the peaks that were greater than 3σ were considered significant. All experimental treatments were replicated 3 times. Data shown are an average of the analyzed data.

RESULTS AND DISCUSSION

In order to obtain the maximum AE output, lyses curves were determined for ml3 and sk1 phage at infection levels of 10^4, 10^5, 10^6 and 10^7pfu/ml (Figure 2 and 3). To amplify the AE affect the maximum cell count was desired, therefore, the lyses of the host should take place just prior to the host entering the stationary phase (control). At 10^4pfu/ml the phage concentration was not sufficient in some cases, that the host could enter into the stationary phase without total lyses occurring, thus an infection level of 10^5 pfu/ml was selected as the desired infection concentration to be used. This infection level allowed for a near maximum AE output to be recorded.

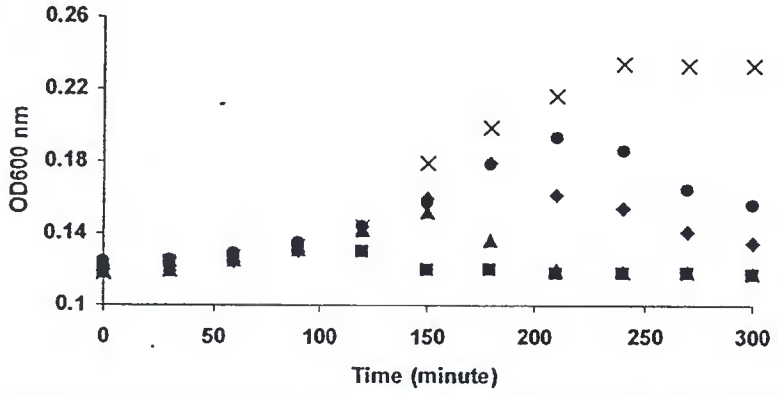


Figure 2. Lyses curves for *L. lactis* ssp. *lactis* C2 when infected with ml3 phage at 10^4 (●), 10^5 (♦), 10^6 (▲), 10^7 (■) pfu/ml and without phage infection (×).

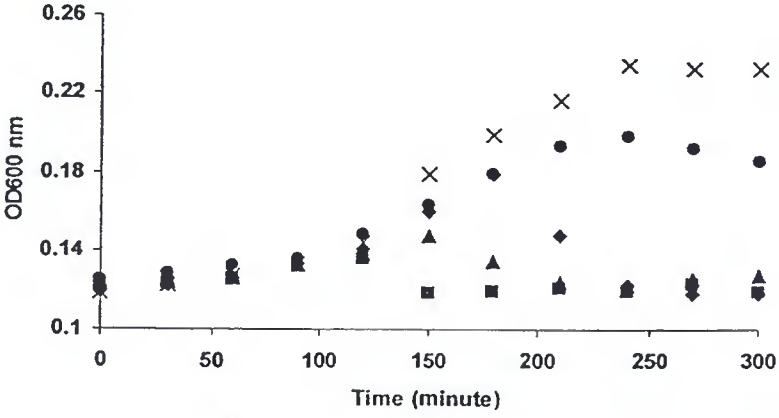


Figure 3. Lyses curves for *L. lactis* ssp. *lactis* C2 when infected with sk1 phage at 10^4 (●), 10^5 (♦), 10^6 (▲), 10^7 (■) pfu/ml and without phage infection (×).

Testing of ml3 and sk1 was performed by using 400 ml of M17 medium at 26°C for 8 h as mentioned in Materials and Methods. When ml3 was added at 90 min to infect the C2 host (Figure 4, purple dash infect line), sensor 1 (Ch1) pick up AE that exceeded the 3σ level whereas sensor 3 (Ch3) where no phage infection performed, recorded no AE that exceeded the 3σ level (level noted by green and red bars as recorded on Ch1 and 3,

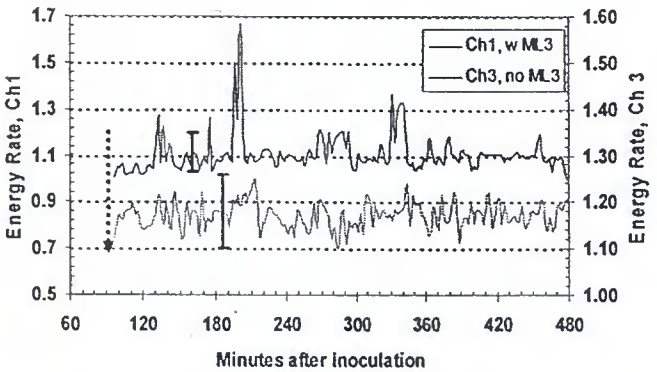


Figure 4. AE signal of *Lactococcus lactis* ssp. *lactis* C2 growth without ml3 phage infection (Ch3) and with ml3 phage infection (Ch1).

We observed that the initial peaks of phage infected cell were 130 and 135 min, the second series of peaks were 190 and 195 min after the first series of peaks, and the third series peaks were 330 and 340 min (Ch1, Figure 4). After 340 min no addition peaks would be expected, which was the case.

The infection cycle of phage has been well studied in *Escherichia coli* (8). It was found that the growth of phage can be divided into 3 periods; first step is phage attachment which is occurred after 10 min. The next step following adsorption of the phage, multiplication occurs for 30 min at 37°C and liberated for the first burst. After 70 min, the phage liberated for the second burst (no visible lysis). This process is repeated leading to a third rise, starting at 120 min, and finally rapid lysis (visible lysis) is occurred for 30 min.

From the current data, it is presumed that the initial infection occurred immediately after the phage was added to the test cell, prior to the data collection system being turned on. Thus, two initial peaks at 40 and 45 min from the time of infection was considered as phage attachment and multiplication. Two larger peaks at 50 and 55 min suggest that second burst occurred prior to the peaks and third peaks at 240 and 250 min after phage injection related to the third burst of phage. During this time the culture would have been moving toward rapid lysis or visible lysis as shown in Figure 5. Thus, after 240 and 250 min we observed that the growth of bacteria was significantly decreased.

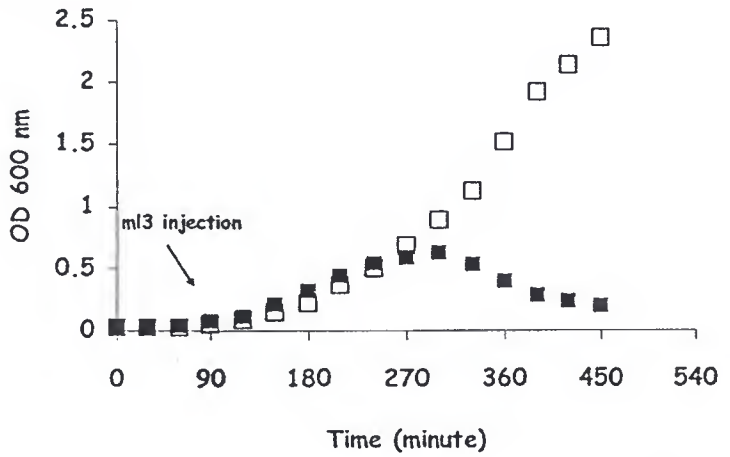


Figure 5. *Lactococcus lactis* ssp. *lactis* C2 growth when infected with ml3 phage (■) and without infected with ml3 (□).

When experiments were run without sk1 phage, no AE that exceeded the 3σ level was observed (level noted by blue and red bars as recorded on Ch1 and Ch3, respectively, Figure 6).

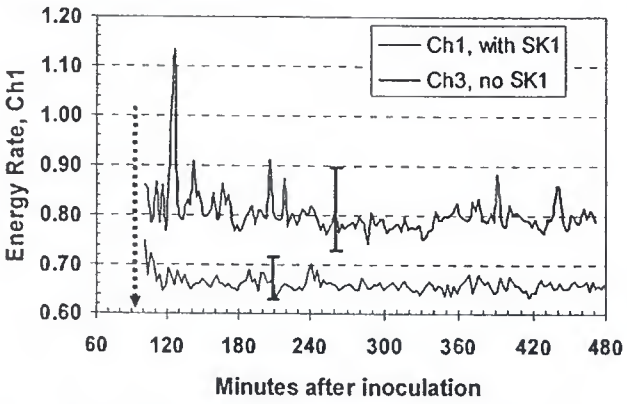


Figure 6. AE signal of *Lactococcus lactis* ssp. *lactis* C2 growth without sk1 phage infection (Ch3) and with sk1 phage infection (Ch1).

However, when the host *L. lactis* ssp. *lactis* C2 was infected with sk1 phage at 93 min after host inoculation into the medium, a significant AE peaks occurred at 126 min, which averaged 33.2 min after infection. All other peaks were less than the 3σ level. When these data were compared to earlier work with c2 phage which had an infection cycle of 38 min (data is not shown), it was apparent that all three phages had different infection cycle times. The different of infection cycle time between three phages might be due to the specific characteristic of those phages. This specificity related to specific recognition between the phage and the host cell (phage attachment or adsorption capacity). In Gram-positive bacteria such as species of lactobacillus phage adsorption almost always involves the cell surface carbohydrates (16, 30). In lactococcus species, phage receptors have been detected on the cell wall material and presumably are part of the peptidoglycan or group-specific carbohydrate (17, 25, 27). It was reported that c2 and ml3 phages adsorb initially to the cell wall and subsequently to host cell membrane protein PIP, which leads to ejection of the phage genome (23, 28). However, phages sk1 adsorption and phage DNA injection into the host does not require a host membrane protein or lipoteichoic acid, and that cell wall components are sufficient for these initial steps of phage infection (10). We assumed that the different requirement for phage attachment will effect the infection time thus leading to produce different profile of acoustic peaks. In the recent data suggested that AE might be a good technique to follow phage infections and provide new strategy for identifying the type of phage.

CONCLUSIONS

AE output from sk1 and ml3 phage could be distinguished from background and external noise. The AE observed appeared to be quite specific for the infection of the host organism, *L. lactis* ssp. *lactis* C2. sk1 phage produced exceeded 3□ peak at 33.2±4.4 min whereas the first peak for phage ml3 appeared 40 min. after infection and that the second infection cycle was 55 min after the first. Thus, this acoustic data collected from phage sk1 and ml3 were considered to be the sound of phage infection the host. AE peak height for second cycle infections compared to first cycle infections were as expected. The timings of the acoustic peaks for phage sk1 and ml3, were sufficiently different that these two phages could probably be distinguished by their acoustic emissions.

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QUESTION AND ANSWER

Question:

a. What kind of medium did you use for proliferating? How well that medium can be used? Have you ever used milk as your medium?
b. Have you ever finded the case in Indonesia?
c. Can you explain the mechanism of acoustic emission signal?

Answer:

a. Good question. I used M17 and I've never used milk

b. *Lactobacillus lactic* infected by bacteriophage in dairy product is the major problem in USA but in Indonesia I don't know. However, this way can decrease the economic cost that is caused from the failure of production.
c. It's hard to explain but I'll try it. I assume that the characteristic of bacteriophage as the shape can affect the adsorption ability of bacteriophage to cell. It also affects the acoustic emission signal.

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