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Antibacterial Activity Test of Bacteriocin from *Lactobacillus brevis, Lactobacillus casei* and *Lactobacillus plantarum* Against Gram Positive Pathogenic Bacteria

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A B S T R A C T

Bacteriocin is a secondary metabolite product of lactic acid bacteria (LAB) which have an antimicrobial and potentially as a natural preservative. LAB isolates used in this study were *Lactobacillus brevis, Lactobacillus casei* and *Lactobacillus plantarum*. This study aimed to determine the antibacterial activity of bacteriocin produced by each isolate of LAB including the influence of pH and heating variation against *Bacillus cereus, Bacillus subtilis* and *Staphylococcus epidermidis*. Antibacterial activity test was done by using disc diffusion method. method. Confirmation test using proteolytic enzyme aimed to analyse that the inhibition zone produced from the activity of bacteriocin. The inhibition zone produced from *L. brevis, L. casei* and *L. plantarum* against *B. cereus* were 15.70, 16.43 and 14.50 mm, against *B. subtilis* were 13.37, 14.10 and 12.53 mm and against *S. epidermidis* were 11.37, 14.50 and 12.45 mm. The activity of each bacteriocin decreased with the addition of trypsin and catalase, bacteriocin was active in the pH range of 2-10 and heating temperature of 40-121^{o}C. Statistical test showed that the addition of trypsin, catalase and the variation of pH also heating had significant differences ($p<0.05$) to antibacterial activity produced by bacteriocin from *L. brevis, L. casei* and *L. plantarum*.

1. Introduction

Foodborne disease that commonly called food poisoning is one of the causes of morbidity and mortality in Indonesia. Some of pathogenic bacteria that related to food poisoning are *Bacillus cereus, Bacillus subtilis* and *Staphylococcus epidermidis*. According to WHO in 2009 incidence rate due to *B. cereus* ≥ 100 cases per 1000 population (Arisman, 2009*). B. subtilis* is the most common bacteria found in 10 out of 24 cutlery samples (41,7%) in Kandou Manado Hospital (Riga et al., 2015). Based on the research (Podkowik et al., 2016), there were 32 *S. epidermidis* isolates that found from 164 samples of ready-to-eat meat products.

Bacteriocin is a secondary metabolite product of lactic acid bacteria (LAB) that has potential as a natural preservative (biopreservative). The advantages of bacteriocin that potentially used as a biopreservative are that it is not a toxic substance, easily degraded by proteolytic enzymes because bacteriocin is a protein compound, does not harm intestinal microflora and stable over wide pH and temperature range (Cleveland et al., 2001). LAB that can produce bacteriocin can be found from fermented food products and processed food products (Palacios et al., 1999).

Es pisang ijo is a Makassar typical drink that is good for health and isolates of LAB that had been identifiedin it was *Lactobacillus brevis* (Syahputria, 2016). *Sotong kering* is a preserved food product in West Kalimantan and LAB isolates that had been identified in itwas *Lactobacillus casei* (Yurinda, 2016). *Ce hun tiau* is a Chinese Pontianak typical drink that has been proven as a producer of LAB and LAB isolates that has been identified in it was *Lactobacillus plantarum* (Malik et al., 2010) (Sari et al., 2016).

The aim of this research was to find out that the antibacterial activity of bacteriocin produced from *L. brevis, L. casei* and *L. plantarum* include the influence of pH and

heating temperature variation against *B. cereus, B. subtilis* and *S. epidermidis* bacteria and confirmation test using proteolytic enzyme aimed to analyse that the inhibition zone produced from the activity of bacteriocin.

2. Materials and Methods

2.1. Materials

Lactobacillus brevis was isolated from *es pisang ijo, Lactobacillus casei* was isolated from *sotong kering* and *Lactobacillus plantarum* was isolated from *ce hun tiau*, de Man Rogosa and Sharpe (MRS) agar medium (Merck), aquadest, crystal violet, iodine, alcohol 96%, safranin, phosphate buffer, NaOH, HCl, trypsin enzyme T880218 (Sigma), catalase enzyme C1345 (Sigma), and Mueller Hinton Agar (MHA) medium (HKM). Bacteria tested in this study were *Bacillus cereus, Bacillus subtilis* and *Staphylococcus epidermidis* that obtained from Biology Laboratory of Faculty of Pharmacy Management Institution, Universitas Tanjungpura.

2.2. Methods

2.2.1. LAB Confirmation

Gram staining was performed by one drop of crystal violet added to the prepared microscope slide which had been smeared with LAB that was isolated for 24 hours then was left for 1 minute and washed with distilled water. Then one drop of iodine was added to the slide and washed with distilled water. The slide was washed with alcohol 96% and then it washed with distilled water. Then, it was added with safranin into the slide and washed with distilled water. The slide contained bacteria was dried and observed by using a microscope (Radji, 2010).

2.2.2. Bacteriocin Production

Each of *L. brevis*, *L. casei* and *L. plantarum* cultures were inoculated into MRS broth then vortexed until homogeneous, after that incubated at 32 ° C for 24 hours. The liquid culture was centrifuged at a speed of 10,000 rpm at 4°C for 15 minutes using a refrigerated micro-centrifugation device. The filtrate was neutralized to pH 7.0 using a pH meter by adding 0.1N NaOH solution. The filtrate was sterilized with bacteria filter with a diameter of 0.22 µm into a sterile tube to obtain an antibacterial supernatant (Usmiati et al., 2007).

2.2.3. Antibacterial Activity Test of Bacteriocin

A 6 mm diameter sterile disc paper was immersed in a bacteriocin supernatant. Disc papers were placed on MHA medium contained test bacteria. The zone diameter produced around the disc paper was measured using a callipers after incubation for 24 hours at 37 ° C (Sidabutar et al., 2015).

2.2.4. Bacteriocin Sensitivity Test against Proteolytic Enzymes

An amount 250 µL of each bacteriocin supernatant from *L. brevis, L. casei* and *L. plantarum* were mixed with 750 µL enzyme with concentration 1mg/mL dissolved in phosphate buffer pH 7.6 for trypsin enzyme and phosphate buffer pH 7 for catalase enzyme then incubated for 24 hours at 37 ° C (Lyon et al., 1991) (Sari et al., 2011).

2.2.5. Effect of pH on Bacteriocin Activity

An amount 5 mL of each bacteriocin supernatant from *L. brevis, L. casei* and *L. plantarum* were put in different tubes, each tube was adjusted to pH 2, 4, 6, 8, and 10 using NaOH or HCl 0, 1 N. After having incubation for 4 hours at room temperature, then bacteriocin activity was tested using agar diffusion method (Kusmarwati et al., 2014).

2.2.6. Effects of Heating on Bacteriocin Activity

An amount 5 mL of each bacteriocin supernatant from *L. brevis, L. casei* and *L. plantarum* were heated at 40, 60, 80, and 100°C for 30 min in waterbath thermostatic and 121°C for 15 min in autoclave (Saad et al., 2015). Then bacteriocin activity was tested using agar diffusion method.

2.2.7. Data Analysis

Inhibition zone diameter data (proteolytic enzyme, pH and heating treatment) were measured using callipers, then each data was analysed using Statistical Program Service Solution (SPSS) using ANOVA test.

3. Results and Discussion

3.1. Gram Staining Results

Gram staining showed that each isolate of bacteria observed (*L. brevis, L. casei* and *L. plantarum*) was Gram positive bacteria characterized by violet colour in bacterial cells. Gram positive bacteria retain the violet colour due to the low lipid content in bacteria when it washed with alcohol, bacterial cell wall more easily hydrated. The hydrated cell wall causes the cell pore size to become small and its permeability decreases therefore the violet colour from crystal violet cannot leave the cell and the cell will remain violet (Assani, 1994). Character morphology was examined are rod shape for those bacteria. This is in accordance with Bergey's Manual of Determinative Bacteriology, the characteristics of the genus *Lactobacillus* are rod-shaped,

Gram-positive and facultative anaerobic (Breed et al., 1957).

Table 1. Bacteriocin inhibition zone diameter

Bacteriocin	Inhibition Zone Diameter (mm)		
	B. cereus	***B. subtilis***	***S. epidermidis***
L. brevis	15.70±0.20	13.37±0.15	11.37±0.15
L. casei	16.43±0.15	14.10±0.10	14.50±0.20
L. plantarum	14.50±0.10	12.45±0.10	12.45±0.10

3.2. Bacteriocin Production

Bacteriocin production was done by inoculating each culture of *L. brevis, L. casei* and *L. plantarum* to MRSB. MRSB is a medium used to produce bacteriocins so that bacteriocins can be released into the medium (Sari et al., 2011). LAB cultures in MRSB were incubated at 32 ° C for 24 hours and will turn turbid due to the release of antimicrobials on the medium. LAB produces the optimum bacteriocin at the incubation time of 24 hours and the maximum incubation time is 48 hours, because at longer incubation times protease or other inactivators can be formed and activated which can reduce the bacteriocin activity. The maximum time in the growth cycle for bacteriocin production depends on the type of bacteria and can occur from the log phase to the initial stationary phase (Hoover et al., 1993).

Table 2. Bacteriocin inhibition zone diameter with the addition of proteolytic enzymes against *B. cereus*

Treatment	Inhibition Zone Diameter (mm)		
	L. brevis	***L. casei***	***L. plantarum***
Bacteriocin Control	15.23±0.25	16.17±0.21	14.50±0.10
Bacteriocin + Trypsin	10.13±0.15	9.35±0.15	10.70±0.20
Bacteriocin + Catalase	11.00±0,20	10.40±0.20	12.17±0.21

The result of centrifugation consists of two layers, namely a clear yellow top layer (supernatant) containing bacteriocin and a lower layer of solids containing bacterial cells. Cell-free supernatant after being centrifuged was measured for pH value by using a pH meter and obtained the initial bacteriocin pH value from *L. brevis* was 4.4, *L. casei* was 5.4 and *L. plantarum* was 4.6. Cell-free supernatant had an acid pH caused by the influence of organic acids which was one of the metabolites of LAB. The acidic supernatant needed to be conditioned into a neutral state at pH 7 with the addition of 0.1N NaOH which aimed to ensure that the inhibitory zone formed was not derived from organic acids but was purely an activity of bacteriocin (Ogunbawo, 2003). The filtrate that had been neutralized then sterilized with a bacteria filter with a diameter of 0.22 µm which purposed to free the supernatant from the remaining bacterial cells due to bacterial cells the remaining ones could contaminate the resulting supernatant. Therefore, the supernatant that had been produced was containing bacteriocin.

Table 3. Bacteriocin inhibition zone diameter with the addition of proteolytic enzyme against *B. subtilis.*

Treatment	Inhibition Zone Diameter (mm)		
	L. brevis	***L. casei***	***L. plantarum***
Bacteriocin Control	13.83±0,25	14.03±0.21	12.53±0.15
Bacteriocin + Trypsin	10.40±0.10	11.33±0,23	7.33±0,15
Bacteriocin + Catalase	11.63±0.15	11.80±0,20	10.30±0.20

3.3 Bacteriocin Antibacterial Activity

Bacteriocin antibacterial test activity purposed to determine the inhibitory activity of bacteriocin from those bacteria against *B. cereus, B. subtilis* and *S. epidermidis*. Bacteriocin is mentioned to have antibacterial activity characterized by the formation of inhibitory zones around the disc. The larger the inhibitory zone is, the greater the bacteriocin inhibition activity against pathogenic bacteria.

Table 4. Bacteriocin Inhibition Zone Diameter with the addition of proteolytic enzyme against *S. epidermidis.*

Treatment	Inhibition Zone Diameter (mm)		
	L. brevis	***L. casei***	***L. plantarum***
Bacteriocin Control	11.80±0.10	14.33±0.15	12.90±0.10
Bacteriocin + Tripsin	8.23±0.15	7.47±0.15	9.43±0.08
Bacteriocin + Catalase	10.20±0.10	10.45±0.15	10.17±0.08

Table 1 bacteriocin antibacterial activity test showed that each of bacteriocin from *L. brevis, L. casei* and *L. plantarum* had antibacterial activity against *B. cereus, B. subtilis and S. epidermidis*. The inhibitory zone formed was irradical zone, which is indicated the activity of the antibacterial is bacteriostatic or inhibits bacterial growth (Syahniar, 2009). The inhibitory zone formed was certainly not came from organic acids because the antibacterial supernatant has been neutralized with NaOH solution. Gram positive bacteria inhibition mechanism by bacteriocinis caused by bacteriocin that attaches to Gram positive bacteria and forms complexes with lipoteichoic acid found on Gram positive bacterial cell wall it can cause destabilization of the cell wall. Lipoteichoic acid is a specific receptor and is

associated with the binding of bacteriocin compounds (Bhunia et al., 1991).

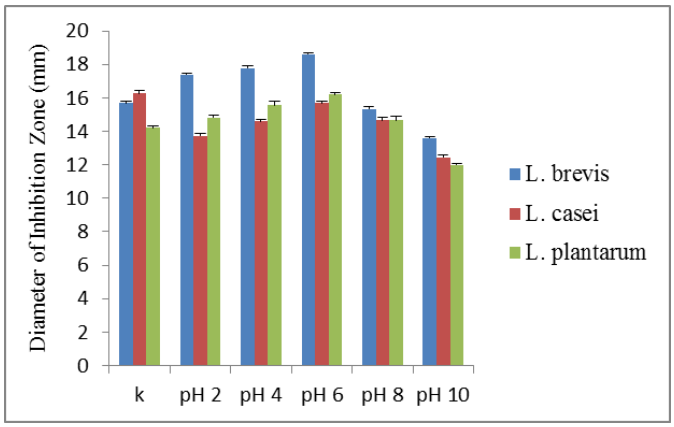

Figure 1. Bacteriocin inhibition zone diameter in different pH conditions against *B. cereus*

According to Ogunbawo (2003), bacteriocin from *L. brevis* OG1 and *L. plantarum* F1 isolated from ogi (Nigerian fermented food) showed antimicrobial activity against some pathogenic bacteria *Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Salmonella typhi* and *Shigella dysentery*. The results were also similar with the study (Mohanty, 2016) reported that bacteriocin from *Lactobacillus casei* (DM 60) isolated from yoghurt showed antimicrobial activity against some pathogenic bacteria in foods *E. coli, B. cereus* and *S. aureus.*

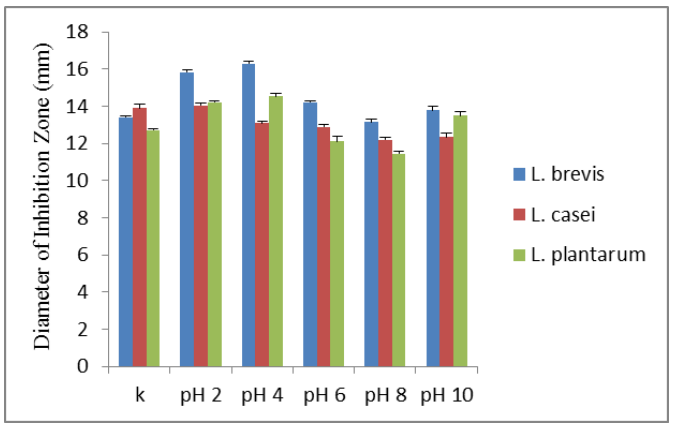

Figure 2. Bacteriocin inhibition zone diameter in different pH conditions against *B. subtilis*

3.4. Bacteriocin Sensitivity to Proteolytic Enzymes

Bacteriocin antibacterial activity using proteolytic enzymes to ensure that the antibacterial supernatant produced by three isolates of LAB is actual bacteriocin. Bacteriocin is a protein which is easily degraded by proteolytic enzymes. The addition of proteolytic enzymes can eliminate the activity of bacteriocin by decreasing or loss of the inhibitory zone around the disc. Proteolytic enzymes will disturb the bonds of amino acids that arrange the bacteriocin that caused bacteriocin activity will be lost and unstable (Parada et al., 2007). Sensibility to proteolytic enzymes evidences the proteinaceous characteristic of bacteriocins (De Martins et al., 2003). Proteolytic enzymes used were trypsin and catalase enzyme.

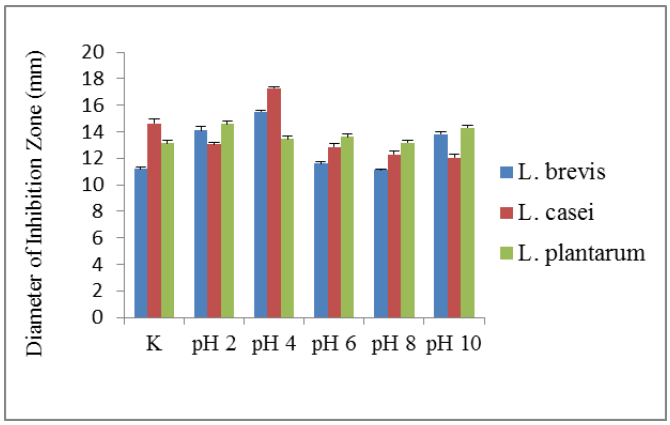

Figure 3. Bacteriocin inhibition zone diameter in different pH conditions against *S. epidermidis*

Tabel 2, 3 and 4 indicated each bacteriocin of *L. brevis, L. casei* and *L. plantarum* antibacterial activity was decrease when added with trypsin and catalase enzyme. Trypsin enzyme had not been able to inactivate bacteriocin from *L. brevis, L. casei* and *L. plantarum*. This could be due to the high active peptides in each of the bacteriocins that can inhibit the growth of *B. cereus, B. subtilis* and *S. epidermidis*. Bacteriocin also has an α-helical structure with opposite polar and nonpolar sides of bacteriocin, making bacteriocin interact with both water and lipid phases when attached to the surface of a sensitive bacterial cell membrane, the cell destabilizes functionally and the cell dies (Ray et al., 2007). The existence of α-helical structure which is still good although bacteriocin has degraded by trypsin enzyme, causing bacteriocin from *L. brevis, L. casei* and *L. Plantarum* still have inhibitory activity against test bacteria. The results similar with the study (Ayuningtyas, 2012) which stated that *plantaricin* inhibitory activity of four purified *L. plantarum* strains still exist although it has been degraded by trypsin enzyme. Based on this result, it can be concluded that the antibacterial supernatant produced by the three isolates of LAB was bacteriocin.

Catalase test were performed to ensure that the antimicrobial activity produced was an activity of bacteriocin and was not activity of hydrogen peroxide. Hydrogen peroxide is one of the metabolites produced by LAB which is also antibacterial. The depletion of the inhibitory zone may

be due to the breakdown of hydrogen peroxide by the catalase enzyme contained in the supernatant so that the bacteriocin produced by the three isolates still contains another metabolite that was hydrogen peroxide (Sari et al., 2011).

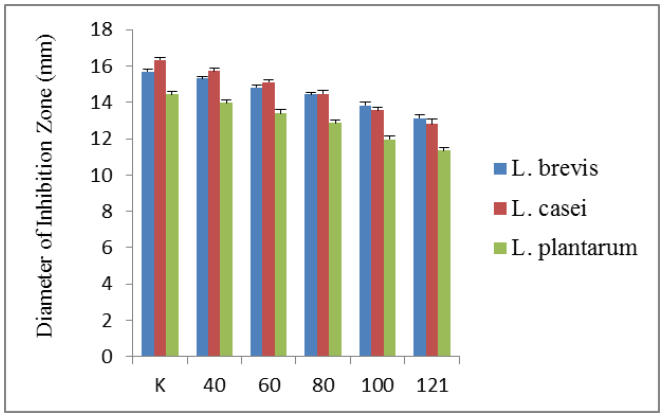

Figure 4. Bacteriocin inhibition zone diameter in different heating temperatures against *B. cereus*

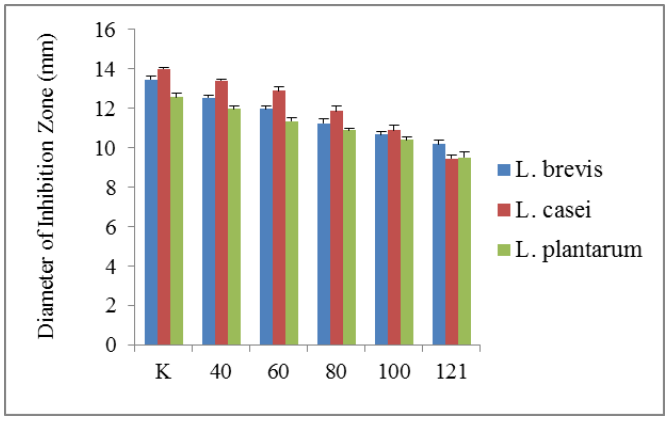

Figure 5. Bacteriocin inhibition zone diameter in different heating temperatures against *B. subtilis*

3.5. Effect of pH on Bacteriocin Antibacterial Activity

Bacteriocin is a protein compound which is antibacterial and used as a preservative. Preservatives that used in the food frequently have taken into consideration because of various factors, one of which is the pH factor. The effect of pH on bacteriocin activity was performed to determine the ability of bacteriocin as antibacterial in various pH range.

Figure 1, 2 and 3 resulted of the pH effect on bacteriocin activity, each bacteriocin of *L. brevis, L. casei* and *L. plantarum* showed antibacterial activity in a wide range of pH (2-10). This could be due to the presence of alkaline amine or carboxylic group of amino acid that arrange the bacteriocin (Balvinder et al., 2011) that caused bacteriocin could retain its antimicrobial activity when there was a shift to acidic or basic range. The results were similar with the study (Gautam, 2009) that reported bacteriocin produced by *Lactobacillus brevis* MTCC 7539 showed antibacterial activity in the pH 3 – 10. Based on the wide range of pH profiles, bacteriocin from *L. brevis, L. casei* and *L. plantarum* are suitable to be used as natural food preservative in acid and base pH such as preservatives in vegetables, fruits, meat, sausage, fermentation in the manufacture of bread and canned food (Buckle et al., 1987).

3.6.Effect of Heating on Bacteriocin Activity

Heating effect on bacteriocin activity was performed to determine the stability of bacteriocin on various heating temperatures. Stability of bacteriocin to temperature is important when bacteriocin will be used as a food preservative because the food production process usually involves heating. The heating temperature used includes 40°C, 60°C, 80°C, 100°C and 121°C.

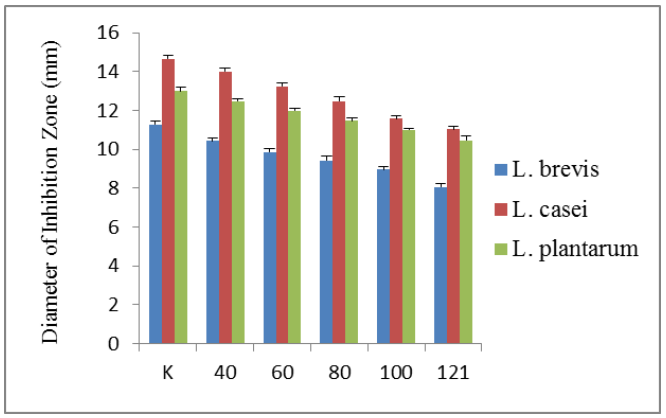

Figure 6. Bacteriocin inhibition zone diameter in different heating temperatures against *S. epidermidis.*

Figure 4,5 and 6 stated that the bacteriocin from *L. brevis, L. casei* and *L. plantarum* showed antibacterial activity subjected heating temperature of 40-100ºC for 30 min to 121°C for 15 min. The heat stability of bacteriocin might be due to formation of small globular form, presence of stable cross-linkages, strong hydrophobic regions and the presence of amino acid cysteine which is able to maintain the bacteriocin structure from heating (Barman et al., 2018) (Nugroho et al., 2003). Similar results reported (Ogunbawo, 2003) that bacteriocin produced by *Lactobacillus plantarum* F1 and *Lactobacillus brevis* OG1 still have activity on heating temperature of 100 º C for 10-30 min and 121 ºC for 10-60 min. The results of (Ullah et al., 2017) stated that bacteriocin *Lin*333 from *Lactobacillus casei* isolated from *Jiangshui Cai* (fermented vegetables from China) was stable at 60-121°C. Based on this character, the three bacteriocin studied can be used as a food preservative, which the process of that food production involves heating.

4. Conclusions

Bacteriocin produced from *L. brevis* was isolated from *es pisang ijo*, *L. casei* was isolated from *sotong kering* and *L. plantarum* was isolated from *ce hun tiau* had antibacterial activity against *B. cereus, B. subtilis* and *S. epidermidis*. The activity of each bacteriocin decreased with the addition of trypsin and catalase. Bacteriocin was active in the pH range of 2-10 and heating temperature of 40-121^{o}C.

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