Isolation and screening of lactic acid bacteria from grasshopper gut as novel probiotic candidates to digest cellulose polymer

R Abdullah, T Erfianti, D A Pratama, and Wijanarka

Department of Biology, Faculty of Science and Mathematics, Diponegoro University, Jl. Prof H. Soedarto SH Tembalang Semarang, 50275, Indonesia
E-mail: ridwanabdulloh20@gmail.com

Abstract. The nutritional needs are the primer need in the earth. Various solution of food security problem such as GMO product and chemical fertilizer, are still not effective to clear it because the global warming annualy reported may cause to the crop failures. In addition, the amount of carbohydrate that can be adsorbed by human body is less. The Cellulose is one of the carbohydrate that human can not adsorb to get glucose. Grasshopper can digest the cellulose of grass because there are bacteria live in their organs to produce the enzyme. The bacteria that have an ability giving advantage in the body are known as probiotic. However, the development of this function has became a great attention. The aim is obtaining grasshopper gut bacteria caracterized as lactic acid bacteria potential as novel probiotic to produce cellulase for human digestion. Methods used are; Isolation of grasshopper gut bacteria, Purification and screening modificated of novel probotic candidates. The bacteria were screened by using modificated medium to detect the enzyme activity and pathogenic possibility as well as SP-SDS method to enumerate the tolerance of the bacteria after treatment in the period. The result shows that two bacteria strains of grasshopper gut bacteria are capable to break down the cellulose in the screening process. The bacteria was also caracterized as the lactid acid bacteria. The activity of gamma haemolytic of the bacteria shows the non-pathogenic property on blood. Bile salt and acid pH condition for 48 hour period was tested on the bacteria shows the high tolerance of life in the digestion. In conclusion, there are strain of grasshopper gut bacteria can be used as novel probiotic candidate to digest cellulose as solution of food security.

1. Introduction

Recently, the Human population growth rate and food needs are going up over the year [1]. Those are not equal with the availabilities of food [2]. Global warming has been reported to be liable as one of the global food scarcity factors [3]. Agriculture land degradation also must be concerned for the next food problem [4]. The most plant on the earth are grasses family. It can live in every extreme region, and may be the best choice to be used as food plant [5]. Nowdays, this plant is not intended for human food, but it is for production of bioethanol. However, all of high plant on the earth are known to contain cellulose or hemicellulose over 40 % [6]. This is the kind of carbohydrate polymer consist of glucose monomers binded by $\beta$ (1→4)-glycosidic bonds [7]. Cellulose polymer of plant also cannot be broken perfectly in human digestion because there is no production of cellulolytic enzymes in human body. It is different in grasshopper digestion system, this animal can digest that polymer because they do symbiosis of mutualism with the microorganisms [8] [9]. The cellulolytic enzyme changes cellulose materials into glucose monomer [8]. Recently, the uses of bacteria that produce cellulolytic enzymes
are still only glued to be used for ruminant animals’ digestion, fuel and textile industry [10]. The safe bacteria that give advantage for the host in digestion is known as probiotic [11]. Probiotics are usually related with genus of lactobacilli bacteria, but the advance research of probiotics has been made recently [11]. The criteria of gut probiotic have been reported in [11], is generally the gram positive bacteria, which can tolerate the pH and bile salt that generally toxic for bacteria 3 for over 2-4 hour. The probiotic also reported must be non-pathogenic to human cell. Some of them may produce anti bacteria for pathogen like E. coli and Staphylococcus aureus.

The aim of the research is obtaining grasshopper gut bacteria in midgut characterized as lactic acid bacteria potential as novel probiotic. This research is using selected bacteria as candidate product for human’s body. It is analysed could be the solution where it aims to increase the ability of human digestion. Cellulose or hemicellulose as the food source in the global food scarcity.

2. Methods

2.1. Bacteria candidate isolation

The grasshopper were collected from Ungaran forest. Valangas nigricornis were living in trees. The preparation of isolation was carried out by making modified De Man Sharpe Rogosa Broth (MRSB) media contain 1% of Carboxymethylcellulose (CMC) [12]. CMC powder is used as cellulose polymer [13]. Isolation was carried out in Laminar air flow (LAF) by washing the samples of Valanga for 4 times aseptically using Alcohol 70% in 5 minutes, aquaest and alcohol will use again 70% in 30 seconds. Valanga were separated between the part of abdomen and the superior of body using a sterile knife. The crude extract collected and it was transferred to microtube contain the media. The crude will be incubated for 4 days in the 120 rpm and temperature condition over 37°C [12]. Then, culture was prepared to transferred in media De Man Sharpe Rogosa (MRS) agar medium contain 1% CMC. Pouring method was carried out by graded dilution technique with oxalate salt in the optimization of Single plate-serial dilution spotting (SP-SDS) method [14]. The bacteria candidates were incubated for 48 hours 37°C in the incubator. Gram staining need to be done after Isolation.

2.2. Cellulolytic test

Isolated bacteria have been tested in the cellulolytic detector media. First, the media must be made by regulating the acidity level pH 3-5 [15]. MRS agar media enriched with 1% of CMC and 0.1% of Congo red reagent [14]. Then, bacteria isolate was transferred to the test media in Laminar air flow (LAF) using streak technique. The bacteria culture was incubated for 48 hours in the incubator with the temperature over 37°C. The grown bacteria with a clear zone after incubation show that the bacteria isolates passed the test. It can be counted for the activity of enzymes by using index of enzyme activity [15].

2.3. Pathogenicity Test

Isolated candidate bacteria from previous result were inoculated into the fresh media. The media is fresh blood contain blood agar base, 5% of type O of human blood, and fresh sheep blood using the streak technique [16]. Human fresh blood and blood agar base were collected from Microbiology laboratory of diponegoro hospital. The bacteria were streaked in the blood media in Laminar air flow (LAF) and incubated for 48 hours at temperature 37 ° C. Then, the isolates that is identified as non-pathogenic bacteria in terms of gamma haemolysis activity, did not show the change of colour in blood media [16]. Probiotics bacteria must be safe to human blood cell without blood lysis action, so that were not harmful when consumed [17].

2.4. Human Stomach Acid and Salt Osmotic Concentration Resistance Test

The candidate bacteria were inoculated into test media before incubated for 48 hours by acid treatment on disk paper, in the condition of pH 7 and pH 2.5. The bacteria was also inoculated into 0.5%, 1%, 1.5% and 2% (w / v) of bile salt solution, then incubated for 48 hour at 37°C [18] and poured into MRS A contained CMC media for 24 hours after carried out using SP-SDS method in plate for counting bacteria concentration after test [19]. The tolerance bacteria were identified by the grown of
bacteria colonies in post-test and counted using the optimization of Single plate-serial dilution spotting (SP-SDS) method.

3. Results and discussion

The various methods of screening assay were carried out to obtained a novel probiotic candidates. Destruction of the abdomen of samples was undertaken by separating the superior parts and crushed it using sterile mortar. The pure bacteria isolate would be obtained without contamination from the other body organ [20]. Figure 1 showed that two bacteria of 80 isolate bacteria were selected from the dilution of 20uL crude, incubating in MRSA medium for lactic acid bacteria screening and gram staining. Both of them showed the morphology of bacteria as probiotic candidate in term of positive gram and the character usually are possessed by bacteria that are able to live in gut condition [18]

![Figure 1](image1.png)

**Figure 1.** The morphology of the bacteria cell potential as novel probiotic from the *Valanga* gut under the microscope at 100x

3.1. Cellulolytic Activity Test

The screening assay was carried out to know the ability of bacteria candidate for cellulase enzyme production. Figure 2 showed that bacteria of strain BAL8G and BAL34G have the clear zone area around the colonies. It can be analysed that the bacteria candidates can convert the cellulose polymer of CMC medium into glucose monomer, and this activity showed by congo-red reagent of the medium. Congo red is useful as an indicator for cellulase enzyme activity released by bacteria [21]. The ability of the cellulolytic activity also derived from the analysis that probiotic bacteria in *valanga*’s colon was useful for obtained the glucose in the body from cellulose polymer [22]. The bacteria candidate must be in acid conditions to hydrolyse the bonding cellulose between glucose monomer [23]. The enzyme activity known is over 1.7 for BAL8G strain and 0.9 for BAL34G strain. It is obtained from the index activity using diameter calculation. Both of the index activity gets near to index 1 and it show the high activity of the cellulase enzyme producing in surrounding.

![Figure 2](image2.png)

**Figure 2.** The Result of bacteria screening in cellulolytic test: a- BAL8G strain isolate ; b- BAL34G strain isolate
3.2. Pathogenicity Test

This screening of pathogenicity test are used to know the pathogenicity activities of the bacteria candidate by analysis of haemolysis [24]. The bacteria candidate strain, BAL8G and BAL34G strain were streaked on blood agar plates medium that are containing 5% human blood. The plates were incubated for 48 hours and temperature condition in 37°C [25]. It was carried out to know the potential in terms of safety for the human as novel probiotics product. Figure 3 showed the results that both of the bacteria strain of BAL8G and BAL34G had no any activity of haemolysis. They showed the positive result with non-pathogenic activity (γ haemolysis). It means that there was no harm of the blood cell, there were no discoloration or the genesis of the clear zones around the colonies of bacteria candidate isolate. From the results, both of the bacteria strains are potentially non-pathogenic for novel probiotic candidates, as in [17]. The γ haemolysis strains are the most favorable candidates’ bacteria for probiotic novel using as product, and it should not be able of producing toxic material that may harmful for the organ surrounding [25].

![Figure 3. The Results of pathogenicity test: a- BAL8G strain isolates; b- BAL34G strain isolates](image)

3.3. Acid and Bile Salt Concentration Tolerance Test

Probiotic should be able to live in the extreme condition, including pH in acid region and bile salt concentration [26] [27]. It was required to kept bacteria active according to the function [28]. Table 4 showed that strain of BALG8 and BALG34 can grow optimize and normal in the region pH 2.5 treatment and with 0.1%, 1%, 1.5% and 2% bile salt concentrations for 48 digestion incubation period. It is strongest bacteria that are able to live in that long period of digestion than others who are only reach up to 4 hours digestion period. Both of the strain was resistance with the high concentration of bile salt, after the treatment and incubation for 48 hours using SP-SDS method. Both of the bacteria isolate grown in testing media by streaking technique, after incubation time in human gut pH condition. It can be analysed that both of bacteria isolates can remain alive in the digestion conditions than other acids, [18]. All of bacteria strains usually had a way to live in different pH indicate no growth at pH 3. It has been known and estimated that the viability rate of probiotics in usual in the host’s gut are only 30%, because the gastric acidity is one of the main barriers [29]. Both of them also have the high population even after the treatment, it showed by SP-SDS colony counting, the bacteria are still having high survival rate over 10⁷ concentration of cell living in CFU/ml. As probiotic, this concentration of living cells is categorized very well to use [30] [31].
Figure 4. Results of acid and bile salt resistance test of the bacteria in the incubation period over 48 hours

4. Conclusion
There are two strains of isolated grasshopper gut bacteria analysed can be practiced in human gut as novel probiotic candidates. The strain is useful to digest cellulose polymer to glucose monomer by producing cellulase enzyme in high activity, safety for human blood and resistance in human gut solvent in digestion period. It can be sustainable for optimizing the activity in living and cellulase enzyme producing.

References
[1] Alexandratos N and Bruinsma J 2012 World agriculture towards 2030/2050: The 2012 Revision ESA Working Paper (Rome: FAO)
[2] Gustavsson J, Caderberg C, Sonesson U, and Otterdijk R V, Meybeck A 2011 Global food losses and food waste – Extent, causes and prevention (Rome: FAO)
[3] Campbell B M, Corner-Dolloff C, Girvetz E, Loboguerrero A M, and Ramires-Villegas J 2016 Global Food Security 11 34
[4] Blum W E H 2013 Int. Soil and Water Conservation Res. 1 1
[5] Sanderson M A and Paul RA 2008 Int. J. Mol. Sci. 9 768
[6] Niemi P, Pihlajaniemi V, Rinne M, and Siika-aho M 2017 Industrial Crops and Products 98 93
[7] Han Y J and Chen H Z 2007 J. Enzyme and Micro. Technol. 41 638
[8] Manhar A K, Bashir Y, Saikia D, Nath D, Gupta K, Konwar B K, Kumar R, Namsa N D, and Mandal M 2016 Microbiological Res. 62 186
[9] Muwawa E M, Budambula N L M, and Osiemo Z L, Boga H I, Makonde H M 2016 African J. Micro. Res. 10 994
[10] Yang W, Meng F, Peng J, Han P, Fang F, Ma L, and Cao B, 2014 Electronic J. of Biotech. 17 262
[11] Halim M, Mustafa N A M, Othman M, Wasoh H, Kapri M R, Ariff A B 2017 LWT - Food Sci. and Technol. 81 210
[12] Huang S, Sheng P, and Zhang H 2012 Int. J. Mol. Sci, 13 2563
[13] Li J, Lewis R B, and Dahn J R 2007 Electrochemical and Solid-state Letters 10 A17
[14] Davis C 2014 J. microbiological methods 103 9
[15] Plessas S, Nouska C, Karapetsas A, Kazakos S, Alexopoulos A, Mantzourani I, Chondrou P, Fournomiti M, Galanis A, Bezirtzoglou E 2017 Food Chemistry 226 102
[16] Ferbiyanto A, Rusmana I, Raffiudin R 2015 HAYATI J. Biosci. 22 197
[17] Yeh E, Pinsky B A, Banaei N, and Baron E J 2009 *PLoS ONE* 4 e6141
[18] Menconil A, Kallapura G, Latorre J D 2014 *Biosci. of Microbiota, Food and Health* 33 25-30
[19] García-Bernal M, Campa-Córdova A I, Saucedo P E, Casanova-González M, Medina –Marrero R and Mazó–Suásteugui J M 2015 *Veterinary World* 8 170
[20] Suman S K, Dhawaria M, Raturi D T V, Adhikari D K, Kanaujia P K 2016 *Int. Biodeterioration & Biodegradation* 112 12
[21] Mamin’ska R T, Szymona K, Madej H, Wong W Z, Bala A, Brutkowski W, Krajewski K, S H’ng P, Mamin’ski M 2015 *Appl. Energy* 160 88
[22] Ni J, Tokuda G 2013 *Biotechnol Adv* 31 838
[23] Fatyasar N I, Chairul I, Primata M, Kang L C. 2015 *J. Solid State Chemistry* 230 163
[24] Zomorodian K, Rahimi M J, Safaei A, Bazzargani A, Motamadi M, Kharazi M, Mostaghni S, Pakshir K, Ghaedi H, Afsarian M H 2011 *J. Microbiol Methods* 85 233
[25] Heindl H, Thiel V, Wiese J and Imhoff J F 2012 *Int. Microbiol* 15 17
[26] Fernandez M F, Boris S and Barbes C 2003 *J. Appl. Microbiol* 94 449
[27] Huang Y, and Adams M C 2004 *Int. J. Food Microbiol* 91 253
[28] Han Q, Kong B, Chen Q, Sun F, and Zhang H 2017 *J. Functional Foods* 32 391–400
[29] García-Bernal M, Campa-Córdova A I, Saucedo PE, Casanova-González M, Medina-Marrero R and Mazón-Suásteugui J M. 2015 *Veterinary World* 8 170
[30] Mokhtari S, Jafari S M, Khomeiri M, Maghsoudlou Y, Ghorbani M 2017 *Food Res. Int.* 96 19
[31] Hook C D, Samsonov V V, Ublinskaya A A, Kuvaeva T M, Andreeva E V, Gorbacheva L Y, Stoynova N V 2016 *J. Microbial Methods* 130 83