AGROKÉMIA ÉS TALAJTAN 72 (2023) 1, 81–94
DOI: 10.1556/0088.2023.00131

The potential of rhizobacteria for use of rambutan (*Nephelium lappaceum*) peel as organic fertilizer

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(Received: 30 Aug 2022; Accepted: 02 Feb 2023)
(First published online: 10 Feb 2023)

Original article

Abstract

Rambutan (*Nephelium lappaceum*) production is growing worldwide so the treatment and utilization of Rambutan by-products has become a concern of manufacturers. The objective of this study was to evaluate the potential application of rhizobacteria to decompose Rambutan peel for organic fertilizer production. After the rhizospheric soil samples were selectively proliferated and preadded on agar medium containing only Rambutan peel, the rhizobacterial colony isolates were screened based on their ability to grow on this agar medium and then to degrade cellulose in Rambutan peel. The LD7.3 isolate from the Rambutan rhizosphere showed the highest efficiency in degrading Rambutan peel with 5.6% degraded cellulose content and was identified by the MALDI-TOF technique as belonging to *Klebsiella*. *Klebsiella* sp. LD7.3 grew well and maintained the same degrading activity after three times of subculturing in liquid medium. Notably, the supplementation of grinded Rambutan fruit peel to the liquid medium had a positive effect on the growth and the degrading activity of *Klebsiella* sp. LD7.3. This was the primary report on the application of rhizobacteria to degrade Rambutan peel and the results showed that this was a potential approach to reuse this waste source.

Keywords: by-product, bio-degradation, *Klebsiella*, organic fertilizer, peel, Rambutan, *rhizobacteria*

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Introduction

The increasing demand for food has led to the accumulation of more and more by-products. In particular, the waste from agricultural cultivation, if not properly treated, will cause the accumulation of organic waste, thus has the risk of adversely affecting the environment. Organic waste from the agriculture are interesting for many researchers to take advantage of, so to increase economic value for agricultural producers. One of the approaches to treatment organic residues from the agriculture has been to convert them to organic fertilizers, in this manner helping to reuse nutrients for plants. In addition, the application of organic fertilizers has been shown to improve the health and structure of agricultural soils.

Rambutan (*Nephelium lappaceum*) is one of the agricultural products with a high proportion in many Southeast Asian countries with many different nutritious food products made from Rambutan fruit. Rambutan has been one of the main fruits exported from countries such as Thailand, Malaysia, The Philippines, Indonesia, and Vietnam. Area and yield of Rambutan tends to increase year by year. Parallel to the growth in production of Rambutan, the amount of fruit peel also increased significantly. The peel and seeds account for 50% of the weight of the Rambutan fruit (MAHMOOD et al., 2018). Rambutan fruit peel have been used to extract antioxidant compounds (MISTRIYANI et al., 2017); for food and non-food applications such as biochar production, fiber, biofuel, etc. (MAHMOOD et al., 2018); and to regulate rumen activity in ruminants (AMPAPON & WANAPAT, 2020).

In addition to valuable biologically active components (aponins, alkaloids, tannins, phytates, and oxalates) as antioxidants, Rambutan fruit peel contain lipids (0.23 g 100 g^{-1}), carbohydrates (23.78 g 100 g^{-1}), proteins (2.04 g 100 g^{-1}), fiber components (0.7 g 100 g^{-1}), and many vitamins such as carotene, thiamine, riboflavin, niacin, and ascorbic acid (MAHMOOD et al., 2018). In particular, Rambutan peel also contain about 1.2% minerals, mainly K, Fe, Zn and Cu (LISDIANA et al., 2019). Previously, there has been no research on the application of bacteria in the degradation of Rambutan peel. If composted, these are essential nutrients for plants in general and Rambutan in particular. However, Rambutan fruit peel have never been an interest as a source in the production of organic fertilizers. Therefore, this study was conducted to evaluate the potential application of rhizobacteria to degrade Rambutan fruit peel towards the production of organic fertilizer.

Materials and methods

Soil samples

Soil samples were taken from the rhizosphere of different crops (including Avocado, Coffea, Lemongrass, Pomelo, and Rambutan) in provinces including Long An (three samples), Lam Dong (seven samples), and Gia Lai (four samples) in Vietnam. Rhizosphere soil was sampled as described by DHAKED et al. (2017). For each sample, after the surface soil layer was removed, about 500 g of soil and roots per tree in the farm was collected by a sterilized shovel. The samples were stored in a sterile zip bag in a cool container and brought to the laboratory.

Rambutan fruit peel

The rambutan fruit peel were supplied by the Co Chin rambutan jam production factory (82, Phu Ninh hamlet, Phu Duc commune, Chau Thanh district, Ben Tre province, Vietnam).

Isolation of rhizobacteria for degrading Rambutan fruit peel

One gram of rhizosphere soil sample was added to 100 ml of the broth (containing g l^{-1}: 1 peptone, 8.5 NaCl, and 5 grinded Rambutan fruit peel) and shaked for 48 h at 200 rpm and 33–35°C. Rambutan peel in this medium was used as the selective substrate. The enrichment culture was diluted and spread on Rambutan peel agar (containing only 5 g l^{-1} of grinded Rambutan peel and 20 g l^{-1}) as a selective medium. After 2–3 days of incubation at 33–35°C, colonies with different characteristics in the plates were purified on Lubria Broth agar. The purified isolates were inoculated on slant Luria Bertani agar to maintain and study further.

Primary screening by the growth of bacterial isolates on Rambutan fruit peel agar

Colony isolates were primary screened for their ability to grow on Rambutan peel agar (the medium used for isolation). Isolates which had the ability to grow well on this medium showed that the isolate had the potential to degrade and use nutrients from Rambutan peel agar. The 2-day-old slant cultures of each isolates were spotted on Rambutan peel agar and incubated at 33–35°C. The experiment was triplicate, each plate was inoculated with three spots. After 48 h of incubation, the growth capacity of the isolates was assessed by the diameter of the bacterial growth zone on the plate.

Screening the potential isolates by degradation of cellulose in Rambutan peel

The 2-day-old slant agar culture of the isolates, which were primary screened, were proliferated in Luria Bertani broth supplemented with 0.5% grinded Rambutan peel at 200 rpm and 33–35°C for 48 h. After proliferation, the culture was diluted by the culture medium for preparation of a bacterial suspension with $OD_{610\ nm}$ = 0.5. 5 ml of the suspension was mixed in 200 g of Rambutan peel (the final humidity was 60%). The mixture of bacterial suspension and Rambutan peel was then incubated at 33–35°C for 25 days. After the incubation period, the cellulose content in Rambutan peel was determined by the improved Scharrer method according to ISO 6541:1981. The experiment was triplicate and the control was the treatment with the culture medium instead of the bacterial suspension. The selected bacterial strain was the one with the best ability to reduce the amount of cellulose in the sample compared to the control.

Identification of the selected isolate by the MALDI-TOF technique

The MALDI biotyper system identifies bacteria/fungi by using the Matrix-assisted Laser Desorption/Ionization (MALDI) technique and the time-of-flight (TOF) analyzer. This technique determines the specific protein mass spectrometry of an organism. The spectra of protein markers are used to identify each particular microorganism by comparing with the spectral library of bacterial/fungal strains

(SINGHAL et al., 2015). The selected isolate were sent to the Center for Science and Biotechnology (University of Sciences, Vietnam National University, Ho Chi Minh City – VNU-HCM) for identification by the MALDI-TOF technique.

Evaluate the growth of the selected strain in liquid media

The selected bacterial strains were cultured for proliferation by inoculating the 2-day-old slant agar culture into Erlenmeyer flask containing Luria Bertani broth and shaking at 200 rpm for 2 days to obtain the 1st culture (*Figure 1*). In the next step, one ml of the 1st culture was inoculated into Erlenmeyer containing 200 ml of Luria Bertani broth supplemented with 0.5% grinded Rambutan peel and cultivated with the same conditions to obtain the 2nd culture. Finally, the same subculturing was conducted for the 3rd culture. The subcultures were determined by the density of cells using the standard plate count technique. The subcultures were also used to degradate Rambutan peel. The enrichment media was used as controls. The experiment was triplicate for each subculture.

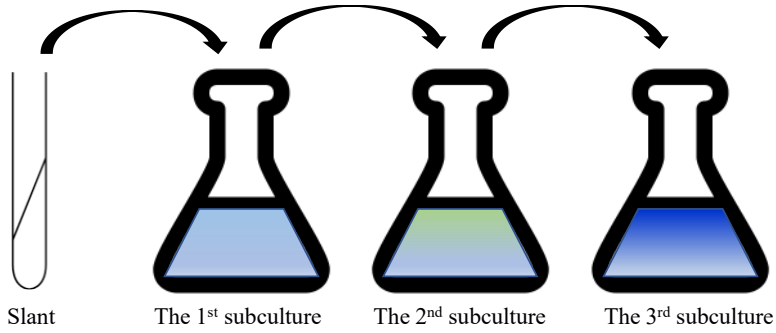

Figure 1
Diagram to evaluate the growth of the selected strain in liquid media by repeated subculturing. Luria Bertani broth was used for the 1st subculture and Luria Bertani broth suplemented 0.5% grinded Rambutan peel was used for the 2nd and the 3rd subculture.

Data analysis

All experiments were arranged in a completely random design. The results were an average of repetitions. Comparisons of means were made using SPSS v.20.1 (IBM, New York, USA) with one-way analysis of variance (ANOVA). Multiple comparisons were determined using the Duncan test at a significance level of $p < 0.05$.

Results and Discussion

Isolation of target bacteria

In this study, 14 soil samples collected in Gia Lai, Long An, and Lam Dong were used to isolate Rambutan peel decomposing bacteria (*Table 1*). Fifty colony isolates were collected after isolation and purification (*Table 2*). In general, all soil

samples showed the presence of target colonies. The number of colonies obtained from each sample varied, ranging from one to seven isolates per sample. In which, the number of isolates obtained from LD3 and LD4 samples in Lam Dong was up to seven isolates per sample, higher than the rest of the other samples. Meanwhile, only one isolate was collected from the LA3 sample collected in Long An. The number of isolates presented in samples from Rambutan rhizosphere in Lam Dong ranged from three to seven. However, the rhizosphere soil of other crops showed only one to five isolates per sample.

Table 1
The collected soil samples

Samples	Provinces	E/N coordinates	Crops
GL1	Gia Lai	13°51’33.4”/107°55’50.7”	Avocado
GL2	Gia Lai	13°51’27.5”/107°55’46.8”	Avocado
GL3	Gia Lai	13°51’28.5”/107°55’31.9”	Coffea
GL4	Gia Lai	13°51’22.4”/107°55’21.0”	Banana
LA1	Long An	10°35’15.8”/106°39’44.8”	Lemongrass
LA2	Long An	10°35’43.2”/106°39’56.8”	Pomelo
LA3	Long An	10°34’15.8”/106°39’31.8”	Pomelo
LD1	Lam Dong	11°83’68.14”/108°21’9.97”	Coffea
LD2	Lam Dong	11°50’15.0”/108°12’40.6”	Coffea
LD3	Lam Dong	11°50’37.0”/108°11’35.5”	Rambutan
LD4	Lam Dong	11°50’49.4”/108°11’27.6”	Rambutan
LD5	Lam Dong	11°51’13.9”/108°12’37.7”	Rambutan
LD6	Lam Dong	11°51’49.5”/108°13’21.4”	Rambutan
LD7	Lam Dong	11°49’54.2”/108°12’59.9”	Rambutan

Table 2
The collected bacterial isolates

Samples	No. isolates	Names of isolates
LA1	4	LA1.1, LA1.2, LA1.3, LA1.4
LA2	3	LA2.1, LA2.2, LA2.3
LA3	1	LA3.1
GL1	5	GL1.1, GL1.2, GL1.3, GL1.4, GL1.5
GL2	2	GL2.1, GL2.3
GL3	2	GL3.1, GL3.2
GL4	3	GL4.1, GL4.2, GL4.3
LD1	2	LD1.1, LD1.2
LD2	4	LD2.1, LD2.2, LD2.3, LD2.4
LD3	7	GL3.1, GL3.2, GL3.3, GL3.4, GL3.5, GL3.6, GL3.7
LD4	7	LD4.1, LD4.2, LD4.3, LD4.4, LD4.5, LD4.6, LD4.7
LD5	4	LD5.1, LD5.2, LD5.3, LD5.4
LD6	3	LD6.1, LD6.2, LD6.3
LD7	3	LD7.1, LD7.2, LD7.3
Total	50	

However, there have been many reports using bacteria in the degradation of other agricultural by-products. For example, GUPTA et al. (2012) isolated bacteria from four different wood-eating animal intestinal samples (termite, snail, caterpillar, and bookworm) and obtained 1–4 isolates per sample that degraded filter paper. ZHANG et al. (2021) also isolated 22 colonies capable of decomposing corn stalks with 1–9 isolates per sample (soil, forest soil, forest humus, decayed wood, and corn stalks). For rice straw, NGO et al. (2021) collected 5 isolates from three different sources of termite intestinal samples with 1–3 isolates per sample. These above reports, as well as many other previous ones, showed that the number of target isolates present at the samples varied widely, depending on the source of the sample used for isolation and the source of the agricultural by-products used for degradation. In this study, the number of target isolates depended on the sampling location. This result indicates that the diversity of target bacteria also depended on crops. The frequent presence of Rambutan residues, including the peel in the soil, could be responsible for the diversity of target bacteria in samples from the Rambutan rhizosphere.

Table 3
The growth of the rhizobacterial isolates on Rambutan fruit peel agar medium

Isolates	The diameter of the growth zones (cm)
GL1.5	0.69^{c}
GL3.1	0.56^{cde}
LD3.2	0.48^{de}
LD4.1	0.53^{cde}
LD4.6	0.67^{c}
LD6.1	0.43^{e}
LD7.3	0.91^{b}
LA2.1	1.11^{a}
LA2.3	0.40^{e}
LA4.1	0.65^{cd}
LA1.1	0.51^{cde}

In the same column, means followed by the same letter(s) indicate insignificant differences at $p < 0.05$.

Screening and identification of target isolates

Thirty nine out of 50 isolates failed to grow on this medium, as indicated by the clustering of biomass at the initial inoculation site, even though all strains were isolated using the same medium. Primary screening showed that there were 11 colony isolates capable of growing on Rambutan peel agar with the growth zone diameters of 0.40 to 1.11 cm (*Table 3*). Among all the isolates, six were isolated from the soil in the Rambutan rhizosphere.

There were strains that could not grow on the media previously used to isolate them. This could be explained by the simultaneous presence of many different colonies in the same isolation disc space and there were interactions that help these colony to grow. According to MOËNNE-LOCCOZ et al. (2014), this type of interaction

between microorganisms living together in the environment is called mutualism, and if not present together, they may not be able to grow. This phenomenon has also been previously reported by many authors. For example, WATSUJI et al. (2006) reported that *Symbiobacterium thermophilum* could not be cultured individually in the absence of *Bacillus* sp. In 2008, JAHN et al. (2007) also demonstrated a similar interaction pattern in two archaea strains, *Nanoarchaeum equitans* and *Ignicoccus hospitalis*.

The frequency of obtaining the target bacterial isolates from the soil of the host crop would be higher than that of other crops. This was probably the reason why studies often used host plant samples to trap or host plant-cultivating soils to isolate target bacteria as previously reported by FISCHER et al. (2007) and recent publications by FOUGHALIA et al. (2022), FENG et al. (2022), ect.

Table 4.
The cellulose content was degraded by the LA2.1 and LD7.3 isolates

Isolates	Degraded cellulose (%)
LD7.3	5.6[a]
LA2.1	1.6[b]

In the same column, means followed by the same letter(s) indicate insignificant differences according to T-test.

Two isolates (LA2.1 and LD7.3) with the remarkable higher diameters compared to nine remaining isolates growing on the medium containing grinded Rambutan peel, were selected to evaluate the ability to degrade Rambutan peel. The analysis results showed that the LD7.3 isolate had higher ability to degrade cellulose in Rambutan peel than the LA2.1 isolate (*Table 4*). In general, the cellulose degrading ability of the two bacterial isolates in this study was lower than that of many previous literatures such as ANBUSELVI & JEYANTHI (2009), DAR et al. (2018), NGUYEN & HOANG (2020). This was probably because of the differences in the natural structure of the contents including cellulose between organic waste sources. In addition, bacterial strain was also one of the important factors contributing to the degradation efficiency. Besides, this result was in contrast to the results of above primary screening through the diameter of the growth zone of these two isolates on Rambutan peel agar (*Table 3*). This could be explained by that the LA2.1 isolate could use Rambutan peel at a low concentration (0.5%) on agar medium for growth, therefore the larger diameter of the growth zone than in the case of LD7.3 isolate. However, Rambutan peel containes phenolic compounds with antibacterial properties (TADTONG et al., 2011). Under the condition of the degradation test with a high content of Rambutan peel, LA2.1 isolate could be inhibited, leading to a lower degradation efficiency than LD7.3 isolate. Meanwhile, LD7.3 isolate was isolated from the rhizosphere of Rambutan, so this isolate could be highly adaptable and unaffected or less affected by the antibacterial compounds present in Rambutan peel during the degradation process. Therefore, LD7.3 isolate showed the highest degradation efficiency under the degradation condition containing a high concentration of Rambutan peel. However, there may be a nutritional deficiency for

the growth of LD7.3 isolate on the agar medium because of the low content of Rambutan peel.

The identification results showed that LD7.3 isolate had a high similarity with bacteria belonging to *Klebsiella*. LD7.3 isolate was characterized as large, round, smooth, mucilaginous, and milky colony on Luria Bertani agar after 2 days of incubation (*Figure 2*). Theese characteristics of LD7.3 isolate on Luria Bertani agar were similar to those of *K. pneumoniae* on the meat peptone agar described by LENCHENKO et al. (2020). However, it could only be concluded that LD7.3 isolate was *Klebsiella* sp. with these morphological characteristics and the Scorevalue of 1,823 by the MALDI-TOF technique. Additional analyzes must be performed in order to identify the species for LD7.3 isolate.

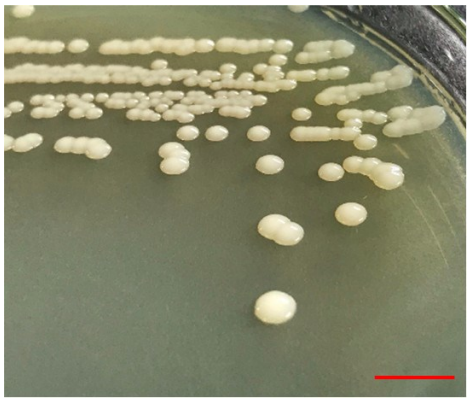

Figure 2
Colonies of *Klebsiella* sp. LD7.3 on Luria Bertani agar after 2-day-incubation. Bar = 1 cm.

Until now, *Klebsiella* has been considered a genus that includes many species that are opportunistic pathogens in humans. However, *Klebsiella* has recently been demonstrated to have an potential application in the degradation of cellulose in various organic waste, as discussed in WAGHMARE et al. (2014), DAR et al. (2018), and BARBOSA et al. (2020). In addition, *Klebsiella* has been the predominant bacteria in the rhizosphere (SACHDEV et al., 2009) and has been able to produce Indole Acetic Acid (IAA) (AHEMAD & KHAN, 2011). *Klebsiella* has also been certified to have other plant growth promoting activities such as nitrogen immobilization (ZEHR et al., 2003; HARINDINTWALI et al., 2021), producing siderophore (AHEMAD and KHAN, 2011; PATTNAIK et al., 2021), improving tolerance to salinity (SAPRE et al., 2018), producing a variety of extracellular hydrolytic enzymes (RODRIGUES et al., 2016), phosphate solubilizing ability, and antagonizing phytopathogens (ZEHR et al., 2003; TILAK et al., 2005; KUMAR et al., 2021). *Klebsiella* sp. PS19 or *K. pneumoniae* M6 strains possessing various plant growth promoting activities have been respectively reported by AHEMAD and KHAN (2011) and KUMAR et al. (2021). *Klebsiella* sp. has been used as biofertilizers in beans, corn, etc. The results in this study once again showed the potential application of *Klebsiella*, and *Klebsiella* was not just

opportunistic human pathogen. However, suitable practice guides are necessary before applying *Klebsiella*.

The growth of Klebsiella sp. LD7.3 in liquid media

Culture media is considered one of the factors that greatly influence the growth of microorganisms. Recently, many authors have chosen plant-based media to culture target microorganisms to reduce organic waste accumulation and production cost. For example, PUTRI et al. (2017) used Pitaya peel extract and CANTABELLA et al. (2021) directly added frozen potato peel and average repulps, tomato seeds after flotation, and wheat bran to the culture medium. Plant-based media have also been suitable for culturing microbial strains in the host plant's own microbiome as discussed by YOUSSEF et al. (2016) and ELSAWEY et al. (2020). Plant-based media have supported cells of rhizospherie microbes to survive longer in the culture and have been recommended for the application for rhizobacteria biomass production.

In this study, *Klebsiella* sp. LD7.3 was subcultured in Luria Bertani broth and Luria Bertani broth supplemented with 0.5% grinded Rambutan peel. The addition of Rambutan peel to Luria Bertani broth made the cell density to increase gradually from the 1st subculture to the 2nd subculture, and 3rd subculture (*Table 5*). *Klebsiella* sp. LD7.3 still maintained the activity of degrading Rambutan peel after three times of continuous subculture in liquid medium. Notably, the efficiency of cellulose decomposition in Rambutan peel of the 2nd and the 3rd subcultures was significantly higher than that of the 1st subculture.

Table 5
The cell density and degradation efficiency of *Klebsiella* sp. LD7.3 subcultures

The subcultures	Medium	Log_{10} (cell density)	Degraded cellulose (%)
1st	LB	9.88^{b}	1.6^{b}
2nd	LB + 5% grinded RP	10.41^{ab}	2.4^{a}
3rd	LB + 5% grinded RP	10.67^{a}	2.7^{a}

LB: Luria Bertani broth; RP: Rambutan peel.
In the same column, means followed by the same letter(s) indicate insignificant differences at $p < 0.05$.

Previously, there have been no studies on culturing bacteria on Luria Bertani broth containing grinded Rambutan peel. YOUSSEF et al. (2016) cultured *K. oxytoca* in the medium containing only cacti (*Opuntia ficus-indica*) and the obtained cell density was significantly lower than those of *Klebsiella* sp. LD7.3 in all of the subcultures. All of the *Klebsiella* sp. LD7.3 subcultures also had a higher cell density than the culture of another strain of *K. oxytoca* grown in the medium containing only slurry homogenates,clover and cactus (MOURAD et al., 2018). *K. oxytoca* also grew well in Berseem Clover and wheat medium; however, cell density data was not available (DAR et al., 2018). The results of this study indicated that the addition of Rambutan peel to Luria Bertani broth clearly had a positive effect

on *Klebsiella* sp. LD7.3 and again confirmed the potential for plant application in microbial growth media.

Continuous subculturing for bacterial proliferation can affect the target activity of bacteria. One of the criteria to evaluate the potential for practical application of a microbial strain is to ensure that cultures after proliferation retains their target activity. The results showed that the supplementation of Rambutan peel to the culture medium, in addition to supporting the growth, also had a positive effect on the target activity of *Klebsiella* sp. LD7.3 after proliferation.

However, this experiment mentioned a lower content of degraded cellulose compared to the strain screening experiment described above (*Table 4*). This phenomenon might be due to differences in the way *Klebsiella* sp. LD7.3 was cultured. In the screening experiment, *Klebsiella* sp. LD7.3 was subcultured in Luria Bertani broth supplemented with Rambutan peel for preparation of the 1st subculture and no preparation of the 2nd and the 3rd subcultures. Meanwhile, in this experiment, *Klebsiella* sp. LD7.3 was grown in Luria Bertani broth for preparation of the 1st subculture and Luria Bertani broth containing Rambutan peel for preparation of the 2nd and the 3rd subcultures. Rambutan peel (containing unknown components) might be essential for the growth as well as for stabilizing the activity of *Klebsiella* sp. LD7.3. However, more studies on the concentration of components (including Rambutan peel and components of Luria Bertani broth) to optimize the culture medium are very necessary.

Conclusions

The *Klebsiella* sp. LD7.3 strain isolated from Rambutan rhizosphere soil was screened from 50 colonies collected from 14 rhizospherie soil samples from various crops. This strain was able to degrade 5.6% cellulose in Rambutan peel. *Klebsiella* sp. LD7.3 had the high adaptability, and the improved growth and target activity after subculturing in Luria Bertani broth supplemented with grinded Rambutan peel. These results are evidence that further strengthen the application potential of *Klebsiella*. This research was a premise for developing microbial products for composting Rambutan peel as organic fertilizer in the future. However, the unresolved problems with *Klebsiella* sp. LD7.3 such as species identification, risk assessment to humans and the environment, determination of suitable conditions for the growth and for decomposing of Rambutan peel, etc. should be studied.

Acknowledgments

The authors would like to thank Van Lang University, Vietnam for funding this work.

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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