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Amylolytic ability of bacteria isolated from termite (*Coptotermes* sp.) gut

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ABSTRACT BSR 2, BSR 3, BSR 8, and BSR 9, different bacteria isolated from the termite gut, have been shown to possess cellulolytic activities, but their amylolytic ability has heretofore been unknown. This study attempted to fill in this knowledge gap. The formation of a clear zone using the iodine test showed that the bacteria were able to produce and secrete amylase. Based on the results, the best cultivation times for strains BSR 2, BSR 3, BSR 8, and BSR 9 were 6, 3, 2, and 2 d, respectively, yielding amylase activities of 2.59 ± 0.13 U/mg, 2.00 ± 0.08 U/mg, 1.67 ± 0.10 U/mg, and 1.55 ± 0.12 U/mg, respectively. BSR 2 had the highest amylase activity compared with the other bacterial isolates. The optimum pH for bacterial amylase activity of BSR 2 was 7.0, and the optimum temperature was 40°C. The molecular characterization of isolates BSR 2, BSR 3, BSR 8, and BSR 9 was based on 16S rRNA gene sequences. Isolates BSR 8 and BSR 9 were thus identified as *Brevibacillus parabrevis* and *Brevibacillus* sp. with similarities amounting to 92.48% and 95.91%, while the BSR 3 isolate was identified as *Pseudomonas alcaligenes* with a similarity of 94.29%, and the BSR 2 isolate could not be identified yet.

KEYWORDS 16 rRNA gene; amylase; amylolytic bacteria; termite gut

1. Introduction

In recent years, enzymes have been widely adopted and exploited in various processes. According to Waites et al. (2001), enzymes are preferred in various catalysis processes because they are environmentally friendly, and can also react specifically so as not to cause undesirable side effects. Amylase is one type of enzyme that has played an important role in the industry. Currently, amylase production has reached a high scale that controls about 25% of the enzyme trade (Reddy et al. 2003). A wide-ranging number of industries utilize amylase, including the paper industry, detergent industry, textile industry, medicine industry, and bakery and cake industries. In Indonesia, production of amylase is still not performed locally, so it must be imported. In fact, locally selected microbes can be used as sources of enzyme production.

Termites consume plant litter and are known to be symbiotic with various kinds of cellulolytic bacteria in their digestive organs. Bacteria originating from their gut have been successfully isolated by Janatunaim et al. (2015) and have been shown to possess a cellulolytic capability. The bacteria from termite guts are also thought to have amylolytic potential, since amylose and cellulose are both linear glucose-based polymers frequently found in plant material, which are distinguished by the glycosidic bonds between the glucose units. The bond in amylose is α-1,4-glycosidic, whereas the bond in the cellulose is β-1,4-glycosidic. Utilization of amylase derived from the termite gut could become an alternative source of a novel amylase. Therefore, it is necessary to conduct a study to prove that bacteria from the termite gut are able to produce amylase, as well as to perform a more detailed characterization of these enzymes to improve their function in the future.

2. Materials and methods

The materials used in this study included: cellulolytic bacteria isolates from the termite gut (BSR 2, BSR 3, BSR 8, and BSR 9) isolated by Janatunaim et al. (2015), nutrient agar medium (beef extract 3 g, peptone 5 g, bakto agar 20 g and prepared in one liter of distilled water), liquid and solid YPSs (Yeast Pepton Starch soluble) (0.2% yeast extract, 0.5% peptone, 0.3% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0,01% $CaCl_2.2\ H_2O$, 2% agar and 2% soluble starch) (Naiola 2001; Soeka 2015), 0.1 M citric acid buffer solution pH 4, 5, 6, and 0.1 M of $CaCl_2.2\ H_2O$, potassium phosphate buffers pH 6, 7, and 8, NaCl, 2N NaOH, K-Na tartate, 3.5 dinitrosalicylic acid (DNS), and aqua dest. Materials for the isolation of DNA consisted of 20 mg/mL proteinase K, 100 mg/mL lysozyme, chloroform, ethanol, lysis buffer, agarose, Tris Borate EDTA (TBE), ethidium bromide, loading dye, DNA ladder 100 bp, Enzymes Star

GLX (TAKARA), 5X PCR buffer, dNTP 2.5 MM, Primary 8F and 1492 R, dd H_2O free nuclease.

2.1. Rejuvenation of bacteria and isolate preparation

The bacteria from the termite gut isolated by Janatunaim et al. (2015) were rejuvenated and grown on CMC medium and YPSs medium for up to three days.

2.2. Detection of amylolytic activity of bacterial isolates from the termite gut

Detection of bacterial amylolytic activity was done through qualitative and semi-quantitative tests. The qualitative test of amylase activity was carried out by the iodine test, while the semi-quantitative test was done by calculating the ratio of the diameter of the clear zone formed with the diameter of bacterial colonies. The result of this calculation is the value of the relative amylase activity.

2.3. Growth of bacteria on liquid YPSs medium

The bacteria from the termite gut were grown in liquid YPSs medium in an incubator shaker at 37°C with a rotation speed of 130 rpm. Furthermore, OD measurements were taken every 24 h for 7 d using a UV-VIS spectrophotometer at 600 nm wavelength.

2.4. Measurement of amylase activity

2.4.1. Isolation of crude enzyme and measurement of amylase activity

Crude supernatant containing amylase of bacteria was isolated by centrifugation. Amylase activity in this supernatant was measured with soluble starch as substrate. The reducing sugars (maltose) released in this reaction were measured by the Bernfeld method (1951) using 3.5-dinitrosalicylic acid (DNS), and its concentration was converted to standard maltose (Bisswanger 2004). One unit of relative amylase activity is the amount of enzymes that can produce 1 µmol maltose per minute per mL of starch solution under test conditions (Bisswanger 2004).

2.4.2. Measurement of protein enzyme levels

Measurement of protein content was done spectrophotometrically with the direct absorbance method at 280 nm wavelength. The standard curve was constructed with BSA, while the blanks used were sterile aqua bidest.

2.4.3. Determination of optimum bacterial cultivation time

Determination of the optimum cultivation time of bacterial strains from the termite gut for producing amylase was done by measuring amylase activity every 24 h for 7 days.

2.4.4. Determination of temperature and pH optimum of amylase activity

The optimum temperature of amylase activity was determined by measuring the activity at various temperatures of 29°C, 37°C, 50°C, 60°C, 70°C, and 80°C using soluble starch as the substrate at pH 7 and incubation for 5 min. The optimum pH for activity was determined by dissolving crude enzyme and soluble starch substrate in 0.1 M buffer solution under various pH conditions, i.e. pH 4, 5, 6, 7, 8, 9, and 10, and incubation at the respective optimum temperature.

2.5. Identification of bacterial strains

Identification was done by the conventional method of DNA isolation, PCR, and sequencing of 16S rRNA gene. Sequences in alignment with the Bioedit program were used for similarity searches in the NCBI database using BLAST.

2.6. Data analysis

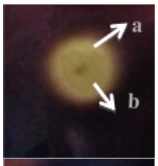
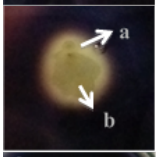
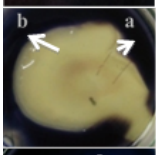
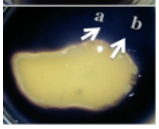
Quantitative data, i.e., bacterial amylase activity, incubation time, temperature, and optimum pH were tabulated and then analyzed statistically using ANOVA and DMRT using SPSS v.16.0.

3. Results and discussion

3.1. Amylolytic activities of bacterial isolates from the termite gut

It was indicated that the four bacterial isolates, i.e. BSR 2, BSR 3, BSR 8, and BSR 9, when grown on YPSs (Yeast Pepton Starch soluble) medium showed positive results in producing amylase, as indicated by the formation of clear zones around the colony (Table 1).

TABLE 1 Qualitative and semiquantitative results of amylase activity.

Bacterial isolates	Results of hydrolysis by amylase	Clear zone diameter: colony diameter (cm)	The appearance of a clear zone around bacterial colonies
BSR 2	+	1.61	
BSR 3	+	1.44	
BSR 8	+	1.04	
BSR 9	+	1.11	

The (+) sign indicates the reaction of hydrolysis of amylose by amylase. The letter *a* in each image shows the clear zone, and *b* denotes the colony.

3.2. Growth of bacterial isolates from the termite gut

Of the bacterial isolates, BSR 9 grew the fastest, followed by isolates BSR 8, BSR 3, and BSR 2 (Figure 1). Based on the analysis (Table 2), it was found that the bacteria growth of all the isolates showed no significant difference ($p > 0.05$) from day one to day two. The growth of isolates BSR 2 and BSR 3 also showed results that were not significantly different across measurement days, except for day 5. Likewise, isolates BSR 8 and BSR 9 showed growth that was not significantly different ($p > 0.05$) to day 7, while the growth of BSR 2 and BSR 9 was significantly different or significant results ($p \leq 0.05$) from day 3 to day 7.

There are several things that can cause a decrease in the number of bacterial cells, such as reduced nutrients available so that some cells experience a decrease in growth rate or die. Gaman and Sherrington (1994) argue that the decline in growth rates can be caused by a lack of growth factors such as vitamins and mineral elements. Measurement of cell growth calculated on the basis of OD values does not generally indicate a decrease in cell growth. This is because the measurement of bacterial OD by spectrophotometric technique not only counts living cells but also dead cells, so that even if all the cells have died, the number of cells counted will still show a constant value because there is no cell growth or cell reduction. However, in the OD measurement results, the data obtained showed a decrease in the number of cells. This may be due to a non-homogeneous sampling, so the number of cells counted from the sample is small.

3.3. Optimum time of amylase production

From the results of the research, it was analyzed that the optimum incubation time of bacterial isolates BSR 2, BSR 3, BSR 8, and BSR 9 for amylase production were 6, 3, 2, and 2 days, respectively, with the respective values for the specific activity of 2.59 ± 0.13 U/mg, 2.00 ± 0.08 U/mg, 1.67 ± 0.1 U/mg, and 1.55 ± 0.12 U/mg (Table 3). The value of amylase activity of all bacterial isolates increased with cultivation time up to the optimum time point, and began to decrease thereafter (Figure 2).

3.4. Effect of temperature on amylase activity

Temperature is an important factor in the enzymatic activity of amylase. In general, the rate of chemical reactions of both enzyme-catalyzed and noncatalyzed reactions will increase with higher temperatures until reaching an optimum. Temperature changes above or below the optimum temperature may result in an increase or decrease in amylase activity (Hmidet et al. 2008). Increasing the temperature to the optimum value will increase the activity of

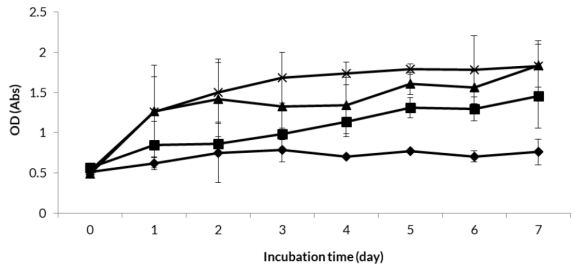


FIGURE 1 Growth curve of bacterial isolates from the termite gut. Standard deviation is calculated from 3 repetitions. (⧫) BSR 2, (■) BSR 3, (▲) BSR 8, (X) BSR 9.

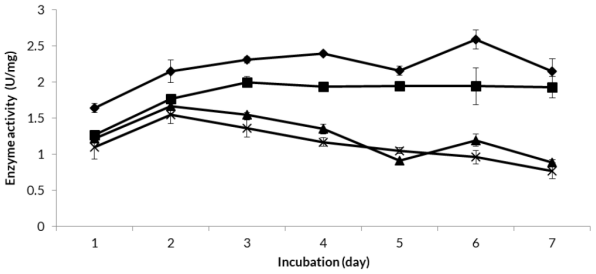


FIGURE 2 Formation of amylase activity during cultivation of bacterial isolates from the termite gut. Standard deviation is calculated from 3 repetitions. (⧫) BSR 2, (■) BSR 3, (▲) BSR 8, (X) BSR 9.

TABLE 2 Average OD values of bacterial isolates from the termite gut every 24 hours for 7 days. Standard deviation is calculated from 2 repetitions.

Incubation (Day)	OD (Abs)			
	BSR 2	BSR 3	BSR 8	BSR 9
0	0.516±0.0^{a,w}	0.562±0.0^{a,w}	0.492±0.0^{a,w}	0.525±0.0^{a,w}
1	0.62±0.083^{a,w}	0.851±0.287^{a,wx}	1.26±0.576^{a,wx}	1.258± 0.438^{a,x}
2	0.751±0.368^{a,w}	0.862±0.087^{a,wx}	1.417±0.502^{a,x}	1.501±0.368^{a,x}
3	0.787±0.151^{a,w}	0.984±0.073^{ab,wxy}	1.326±0.346^{ab,wx}	1.686±0.314^{b,x}
4	0.703±0.006^{a,w}	1.138±0.184^{ab,xy}	1.34±0.351^{bc,wx}	1.74±0.142^{c,x}
5	0.771±0.008^{a,w}	1.313±0.127^{b,xy}	1.612±0.134^{c,x}	1.79±0.067^{c,x}
6	0.706± 0.071^{a,w}	1.297±0.15^{ab,xy}	1.565 ± 0.201^{b,x}	1.781±0.429^{b,x}
7	0.76±0.163^{a,w}	1.455±0.401^{ab,y}	1.83±0.269^{b,x}	1.83±0.319^{b,x}

$^{a-c}$ Different notations on values in the same line indicate a significant difference at the level of significance 5% ($p \leq 0,05$). $^{w-y}$ Different notations on values in the same column indicate a noticeable difference at the level of significance 5% ($p \leq 0,05$).

TABLE 3 Mean of amylase activity formed by bacterial isolate from the termite gut. Standard deviation is calculated from 3 repetitions.

Activity (U/mg)	Isolates			
	BSR 2	BSR 3	BSR 8	BSR 9
1	1.64±0.06^{b,w}	1.27±0.01^{a,w}	1.214±0.047^{a,w}	1.099±0.165^{a,wx}
2	2.15 ±0.15^{c,x}	1.77±0.02^{b,x}	1.667 ±0.1^{ab,z}	1.551±0.124^{a,z}
3	2.31±0.04^{d,xy}	2.00±0.08^{c,x}	1.55±0.032^{b,y}	1.36±0.118^{a,y}
4	2.39±0.0^{d,y}	1.94±0.04^{c,x}	1.350 ±0.07^{b,x}	1.168±0.067^{a,x}
5	2.16±0.06^{d,x}	1.95±0.06^{c,x}	0.907±0.015^{b,v}	1.0513±0.045^{a,wx}
6	2.59±0.13^{c,z}	1.95±0.26^{b,x}	1.193 ±0.084^{a,w}	0.959±0.093^{a,vw}
7	2.15±0.18^{b,x}	1.93±0.15^{b,x}	0.882±0.042^{a,v}	0.769±0.111^{a,v}

$^{a-c}$ Different notations on values in the same line indicate a noticeable difference at the level of significance 5% ($p \leq 0{,}05$). $^{w-y}$ Different notations on values in the same column indicate a noticeable difference at the level of significance 5% ($p \leq 0{,}05$).

enzymes in forming the product, while a temperature exceeding the optimum may cause damage or denaturation of the enzyme.

As shown in Figure 3 and Table 4, the optimum temperature of amylase activity was at 40°C with an activity value of 2.59 ± 0.13 U/mg, which was significantly different ($p \leq 0.05$) from the amylase activity at other incubation temperatures. Based on the analysis, it was found that amylase activity at 50°C to 80°C did not show any significant difference ($p > 0.05$), meaning that a temperature change in the range of 50-80°C did not greatly disturb enzyme activity.

The results of the research showed data in accordance with Mishra and Behera (2008), who reported that bacterial isolates exhibiting amylase activity have high amylase activity at 40-45°C, especially *Bacillus* species. The results also concurred with a study by Gebreselema (2015), which found the optimum temperature for amylase activity to be 40°C in *Bacillus* and 37°C in *Streptomyces*.

3.5. Effect of pH on amylase activity

The pH value greatly affects the shape of the enzyme ion structure which can be positive ions, negative ions or double charged ions (zwitter ion). With the change of pH conditions it will affect the effectiveness of the active site of the enzyme in forming the enzyme-substrate complex. When the pH conditions are too acidic or too alkaline from the optimum pH conditions of the enzyme, enzyme denaturation may result in a decrease of enzyme activity, even it may completely be lost (Poedjiadi 1994).

The amylase of bacterial isolate BSR 2 showed its optimum activity at pH 7. As Figure 4 and Table 5 show, amylase activity continued to increase as the pH increased from acidic to neutral, i.e. pH 4 to 7, and then showed a decrease when going into the alkaline region, i.e. pH 8 to 10. These results suggest that the optimum pH to obtain the best amylase activity of bacterial isolate BSR 2 from the termite gut is pH 7. That is, amylase from BSR 2 bacteria will perform optimally at a neutral pH condition.

The amylase activity calculated at pH 7 was significantly different ($p \leq 0.05$) compared with the amylase activity at other pH levels, except at pH 6, which places the acidic atmosphere close to neutral. This is in accordance with Baker (1983), who stated that amylase derived from insects is generally active in neutral to slightly acidic pH conditions (Terra et al. 1996), since bacterial isolates used in this study also come from insects, namely termites. Vihinen and Mantsiila (1989) also confirmed that the optimum pH of amylase varied from 2 to 10.5, but most were well active at pH 5-8. The results of this study were

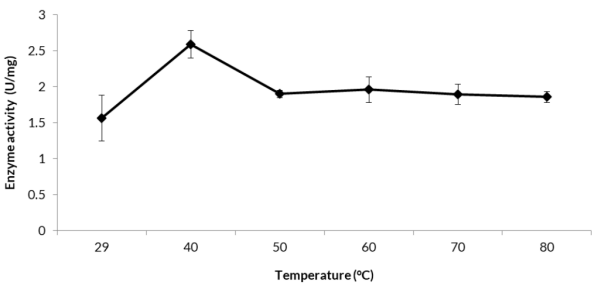


FIGURE 3 Results of measurement of amylase activity of bacterial isolate BSR 2 on temperature variation. Standard deviation is calculated from 3 repetitions.

TABLE 4 Mean of amylase activity of bacterial isolate BSR 2 on temperature variation. Standard deviation is calculated from 3 repetitions.

Temperature (°C)	Amylase activity (U/mg)
29	1.57±0.31^{a}
40	2.59±0.13^{c}
50	1.90±0.05^{ab}
60	1.96±0.18^{b}
70	1.89±0.14^{ab}
80	1.86±0.08^{ab}

$^{a-c}$ The difference in notation on the values in the same column shows a significant difference at the 5% significance level ($p \leq 0.05$).

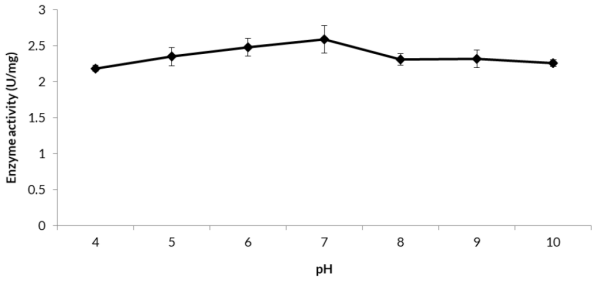


FIGURE 4 The results of the measurement of bacterial isolation activity of BSR 2 on the pH variation. Standard deviation is calculated from 3 repetitions.

TABLE 5 The mean of amylase activity of bacterial isolate BSR 2 on pH variation. Standard deviation is calculated from 3 repetitions.

pH	Amylase activity (U/mg)
4	2.19±0.04[a]
5	2.35±0.13[ab]
6	2.48±0.12[bc]
7	2.59±0.13[c]
8	2.31±0.08[ab]
9	2.32±0.12[ab]
10	2.26±0.05[a]

[a-c] The difference in notation on the values in the same column shows a significant difference at the 5% significance level ($p \leq 0.05$).

also supported by the results of Mishra and Behera (2008) and Alariya et al. (2013), both of whom reported that the best amylase activity of *E. coli*, *Pseudomonas fluorescens*, *Bacillus subtilis*, and *Serratia marscens* bacteria occurred at pH 7.

In pH conditions that are too acidic or too alkaline, the activity of amylase will generally be slower and decrease, as stated by Pujawati (2012), that the pH of the environment affects the speed of enzyme activity in catalyzing a reaction (Abdel-Fattah et al. 2013). The results of Hmidet et al. (2008) also showed that enzymes that are given a pH treatment that is too alkaline (pH 10) could result in protein denaturation and total loss of activity. However, from the results of this study it was found that the amylase of isolate BSR2 had activity that was not significantly different ($p > 0.05$) when it was treated at an acidic pH level (i.e. 4 and 5) as well as the pH bases of 8, 9, and 10. These data suggest that amylase from BSR 2 is able to tolerate a wide range of pH.

3.6. Identification of the bacterial isolates

Amplification of the 16S rRNA genes of bacterial isolates BSR 2, BSR 3, BSR 8, and BSR 9 was performed (Figure 5). The sequences of the 16S rRNA genes of bacterial isolates BSR 8 and BSR 9 showed the highest level of similarity with those of *Brevibacillus* sp. AB938197.1 (Table 6). *Brevibacillus* spp. are Gram-positive bacteria (Thomas 2006), and most of the *Brevibacillus* bacteria are

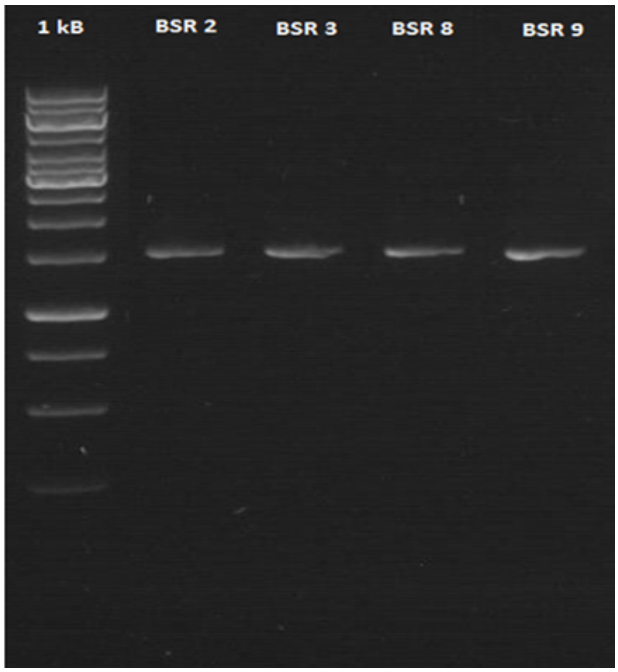


FIGURE 5 Results of amplification of the 16S rRNA genes of bacterial isolates BSR 2, BSR 3, BSR 8, and BSR 9, which after purification gave a single band at 1500 bp.

isolated from the environment, especially from the soil, and are also commonly found in milk and cheese (Vos et al. 2009). The bacterium isolated from the termite gut, BSR 3, showed the highest similarity level with *Pseudomonas alcaligenes*, at 94.29%. *Pseudomonas* spp. are generally facultative aerobic but do not form spores. Common features of the genus *Pseudomonas* are Gram negative, obligate, motile, negative fermentative oxidation (Cornelis 2008). *Pseudomonas* can be isolated from soil or water. Silaban (1999) successfully cultivated *Pseudomonas alcaligenes* in CMC (carboxymethyl cellulose) media which showed that the bacteria had the ability to degrade cellulose. Nevertheless, no studies have shown that *Pseudomonas*, especially *Pseudomonas alcaligenes*, can be isolated from termite gut. As for the BSR 2 isolate, it only shows a low similarity level with the existing data in genBank so it can be said the identification of the type of bacterial isolate is not yet possible. The highest value of conformity is with *Pseudomonas* sp. and *Stenotrophomonas maltophilia* but only below 60% with a 36% query cover score.

Based on various studies of cellulolytic bacteria in termite digestive tracts, the bacteria identified were *Bacillus megaterium* and *Paracoccus yeei* from a study conducted by Ferbiyanto et al. (2015). The study was conducted on cellulolytic bacteria isolated from the digestive tract of *Macrotermus gilvus*. A similar study was conducted by Wenzel et al. (2002), who successfully isolated cellulolytic bacteria from the digestive tract of *Zootermopsis angusticollis*. The cellulolytic bacteria were identified as *Cellulomonas*, *Bacillus cereus*, *Bacillus megaterium*, and *Paenibacillus* species.

TABLE 6 Results of BLASTN of the sequence of the16S rRNA genes and similarity analysis.

Isolates	Description of BLAST analysis	Accession number	% similarity
BSR 2	*Stenotrophomonas maltophila* SSASC23	KM875660.1	60.51
	Pseudomonas sp. B1	KJ855516.1	67.44
BSR 3	*Pseudomonas mendocina* strain PC19	DQ178226.1	93.67
	Pseudomonas alcaligenes strain NCIMB9867	JX867714.1	94.29
BSR 8 and BSR 9	*Brevibacillus parabrevis* strain M3	AB215101.1	92.48
	Brevibacillus sp. IICDBZ12	JN836929.1	95.91
	Brevibacillus brevis	KM191288.1	95.68

4. Conclusions

Bacterial isolates BSR 2, BSR 3, BSR 8, and BSR 9 from the termite gut can produce amylase, and best amylolytic bacterial isolate is BSR 2. The optimum bacterial cultivation time of BSR 2, BSR 3, BSR 8, and BSR 9 for producing amylase were 6 d, 3 d, 2 d, and 2 d; respective amylase activity values were 2.59 ± 0.13 U/mg, 2.00 ± 0.08 U/mg, 1.67 ± 0.1 U/mg, and 1.55 ± 0.12 U/mg. The optimum conditions for the amylase activity of bacterial isolate BSR 2 from the termite gut was at 40°C and pH 7. BSR 8 and BSR 9 were identified as *Brevibacillus parabrevis* and *Brevibacillus* sp. based on similarities of the sequence of the 16S rRNA genes (92.48% and 95.91%), BSR 3 was identified as *Pseudomonas alcaligenes* with similarities amounting to 94.29%, while the BSR 2 isolate could not be identified yet due to low sequence similarities.

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Authors' contributions

PDM, RMH, RZJ, YAP designed the study. PDM, RMH carried out the laboratory work. PDM, RMH, RZJ analyzed the data. PDM, RMH, RZJ wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare no competing interest.

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