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Phenetic Diversity of Cellulase-Producing Bacteria from Wana Tirta Kulon Progo Mangrove Forest

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ABSTRACT

Mangrove is an ecosystem that have important value for the environment and are a source of cellulase-producing microorganism biomass. The purpose of this study was to determine the characteristics and types, as well as the highest cellulase enzyme activity from cellulase-producing bacteria found in Wana Tirta Mangrove Forest, Jangkaran, Kulon Progo. This research is a descriptive-exploratory research. Sampling in the form of litter, mud and water was carried out in the Wana Tirta Kulon Mangrove Forest which was divided into 3 plots. This sample is then isolated on selective media in the form of Carboxymethyl cellulose (CMC) In order to grow cellulolytic bacteria. The bacteria obtained are then purified and phenetic characterization. The data obtained were used to classify the bacterial isolates using MVSP identification software (Multivariate Statistical Package) 3.1 with UPGMA clustering algorithm (Unweight Pair Group Method With Arithmatic Averages) then the result is presented in the form of a dendogram. Result Research shows as many as 17 isolates of cellulolytic bacteria were obtained. There were 4 bacterial isolates from litter samples, 1 bacterial isolate from water samples and 12 bacterial isolates from mud samples. After being made in the form of a dendogram, 19 bacterial clusters were obtained. A total of 6 bacterial isolates had a similarity index of ≥ 72% against Bacillus pumilus namely isolates S1A 2, S2A, AL 3, AP 3, AP 6 and AP 17, 4 bacterial isolates have a similarity index of ≥ 70% to Bacillus stearothermophyllus namely AP 22, AP 24, AP 25, and AP 26 isolates. As well as 7 bacterial isolates have a similarity index of ≥ 77% against Streptomyces Sp. namely isolates S1A 1, S3B, AP 8, AP 9, AP 14, AP 20 and AP 27.The highest cellulase enzyme activity occurred in AP 14 bacterial isolate of 286.72 U/ml.

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1. INTRODUCTION

Cellulase enzyme is an enzyme complex that decomposes cellulose into glucose by hydrolyzing β-1,4 bonds in cellulose (Budi, 2018). Cellulase enzymes produced by microorganisms are currently the focus of biocatalysts due to their complex nature and application in the vast industrial world (Kuhad et al., 2011). In addition, cellulase enzymes are also used in pharmaceuticals, medicine, genetic engineering, protoplast production, analytical applications, and pollution handling (Sharada et al., 2014). This cellulase enzyme degrades cellulose molecules into monosaccharides or simple disaccharides so that they can be used by microorganisms to support their lives (Hasanah, 2015).

Bacteria capable of secreting cellulase enzymes are cellulolytic bacteria. Cellulolytic bacteria can naturally be found in mangrove forests, agricultural land, and on decaying plant tissues (Azizah, 2013). Mangrove forests are ecosystems that have high productivity compared to other ecosystems. In this ecosystem there is a high decomposition of organic matter, thus making the forest ecosystem mengrove into a place rich in food sources and habitats for various kinds of biota such as fish, crabs and shrimp (Kurniawan et al, 2018). The overhaul of organic matter in mangrove ecosystems occurs due to the activity of microorganisms that are able to produce extracellular enzymes in the form of cellulase enzymes by cellulolytic bacteria (Zulaika et al., 2022, Wijiyono, 2009).

The coastal area of Kulon Progo, which is directly adjacent to the Indian Ocean and is the estuary of three rivers, namely the Progo River, Bogowonto River, and Serang River, has a high mangrove growth potential of 95% (Pintari, 2019). This indicates the diversity of cellulolytic microorganisms that play a role in the remodeling of various organic materials in the region.

One of the mangrove forest areas in the area is the Wana Tirta Mangrove forest located in Jangkaran Village, Temon District, Kulon Progo Regency. In the mangrove forest, there have not been many studies in the field of microbiology. Research conducted by Pintari (2019) in the area is limited to studying cellulolytic fungi while studies on cellulolytic bacteria have not been carried outn. This underlies the need for research on cellulase-producing bacteria in the region as a new source to obtain cellulase enzymes that are widely used in the industrial world.

The large use of cellulolytic enzymes in various fields causes the need to conduct extensive studies on the sources of cellulolytic enzyme production so that the need for this enzyme can be met. Seeing the large potential of mangrove forests as a source of cellulase-producing biomass causes the need for isolation and identification of cellulolytic bacteria obtained from these mangrove forests in the hope that the bacteria found can be a new source as a producer of cellulase enzymes. The identification of these bacteria can be done with a phenotypic character approach through observations of colony morphology, cell morphology, physiological (biochemical) properties and the influence of environmental factors. On this basis, in this study, phenotypic character observations were made on acquired bacteria. These phenotypic characters will later become the basis for making dendograms using analytical methods Simple Profile Matching. The dendogram will show the diversity of cellulase-producing bacteria isolated from the Wana Tirta Jangkaran Kulon Progo mangrove forest. The results of this study can later be used as a reference or reference for future research that examines the diversity of seulolytic bacteria in the mangrove forest area and its potential as a producer of cellulase enzymes which are widely used in various fields. The research conducted by this author aims to determine the phenetic diversity of cellulolytic bacteria from the Wana Tirta Kulon Progo Mangrove Forest along with the activity of cellulolytic enzymes produced.

2. RESEARCH METHOD

2.1. Types of Research

This research is a type of exploratory descriptive research with the object of research in the form of cellulolytic bacteria isolate from Wana Tirta Kulon Progo Mangrove Forest.

2.2. Time and Place of Research

This research was conducted in February - May 2021. Sampling was carried out in the Wana Tirta Kulon Progo Mangrove Forest. Isolation, characterization and identification of cellulolytic bacteria were carried out at the Microbiology Laboratory, FMIPA UNY.

2.3. Population and Research Sample

The study population included all cellulolytic bacteria in the Wana Tirta Kulon Progo Mangrove Forest. Research samples in the form of cellulolytic bacteria from samples in the form of litter, water and mud.

2.3. Procedure

2.3.1. Location determination

Determination of sampling location using the method *purposive sampling*. According to Sugiyono (2008) *purposive sampling* is a sampling technique as a source of data carried out with certain considerations (Mukhsin, 2017). In this study, the determination of the location of data

collection in mangrove areas was based on the presence of mud, leaf litter and water that has the potential to be a source of cellulolytic bacteria. This sampling location is divided into 3 plots.

2.3.2. Sterilization of tools and materials

In this study, the sterilization process was carried out using *an autoclave* , which is a sterilization technique with pressurized hot steam. *Autoclaves* are used to kill microorganisms with a pressure of 1 atm, at a temperature of 121º C for 15 minutes (Cowan and Steel, 1993 in Sinatryani, 2014). The sterilization stage begins with washing equipment using detergent and running water. Then the whole equipment is dried. The holes contained in the equipment are corked using caps and aluminum foil. Then the equipment is wrapped in heat-resistant plastic and tightly fastened before being inserted into the *autoclave*.

2.3.3. Sampling

The samples taken were in the form of mud, leaf litter and water. This sample is put in a container that has been given a naming code then put in a *cooling box,* then taken to the Microbiology Laboratory FMIPA UNY.

2.3.4. Isolation and purification of cellulolytic bacteria

Isolation of cellulolytic bacteria is carried out by the method of *Spread Plate* on selective media in the form of CMC Agar. Samples in the form of mud weighed as much as 1 gr then mixed in 9 ml of sterile aquades and then vortex to be homogeneous. This mixture is a dilution of 10^{-1}. Then as much as 100µl of the mixture is planted on CMC Agar media and incubated. In samples in the form of water, planting is carried out without dilution because the water obtained is in fairly clear condition. A total of 100µl of water sample was planted on a spread plate on CMC Agar media and then incubated. In samples in the form of leaf litter, planting was carried out by technique *direct plating*. This is done with the aim of obtaining diverse bacterial isolates. Litter samples are cut with a size of approximately 1x1 cm then planted on CMC Agar media and then incubated. The growing bacterial colonies are then inoculated on Nutrient Agar media by scratching method. All these isolation measures are carried out aseptically. Bacterial colonies grown from the entire sample planting were then selected to obtain different bacterial isolates. The bacterial isolate is then purified into one nutrient agar slant media containing only one type of bacterial isolate.

2.3.5. Characterization and identification of bacteria

All isolates of cellulolytic bacteria to be characterized are pure isolates of 24-hour-old cellulolytic bacteria except for painting pure culture endospores of 72-hour old bacteria (Chasanah, 2018). In this study, phenetic characterization was carried out in the form of gram staining testing, endospore staining, colony morphology observation, oxygen demand, gelatin hydrolysis, carbohydrate fermentation, cellulolytic index (IS), amylum hydrolysis, catalase, citrate, H2S production, indole production, *Methyl Red (MR), Voges-Proskauer (VP),* motility, influence of temperature, influence of pH, as well as activity of cellulase enzymes.

2.4. Data Collection Techniques

The primary data in this study is in the form of data from observations of isolates Cellulolytic bacteria in the form of phenotypic characters and cellulase enzyme activity, and secondary data by examining theories and information from various relevant sources (journals, papers, books, theses, websites).

2.5. Data Analysis Techniques

Data obtained from a wide variety of tests are included in the table and used as a basis for determining bacterial kinship. The data used to make a dendogram is the result of characterization of bacterial isolates and coupled with testing data from research articles in scientific journals and strain type Unpredictable species. The test result data is arranged in the form of an nxt table in the MS Excel program. The Operational Taxonomicical Unit is n inserted as a column while the phenotypic character of bacteria is t inserted as a row. The encoding in the table uses a binary system, namely the notation 1 if the test result of a certain character is positive while 0 if the test result is negative. The

construction of the dendogram was performed using the Multivariate Statistical Package (MVSP) 3.1. The program requires input in .mvs format which can be generated using the PFE (Programmer's File Editor) program or Notepad. Determination of the similarity of isolates using coefficients Simple Matching Coeficient (SSM). This grouping is done with the UPGMA algorithm then the results are presented in the form of a dendogram (Sembiring, 2002 in Chasanah, 2018).

3. RESULTS AND ANALYSIS (11 Pt)

This research began with the sampling stage of isolate sources carried out in the Wana Tirta Mangrove Forest using the method *purposive sampling*. Method *purposive sampling* is a sampling technique as a source of data carried out with certain considerations (Sugiyono, 2008 in Mukhsin, 2017). Sampling was carried out in 3 plots shown in Figure 1.

3.1. Qualitative test of cellulolytic bacteria

The result of this qualitative test of cellulolytic bacteria is the value of the Cellulolytic Index (IS). This test was carried out by dripping congo red on bacterial isolates that had been grown on CMC Agar media. Congo red serves to clarify the clear zone formed. Anand *et al*. (2009) said that *Congo Red* will bind specifically to polysaccharides that have β-1,4 glycoside bonds which are polysaccharides contained in CMC Agar media. The appearance of the clear zone after being dripped with *congo red* is shown in Figure 2.

Figure 1. Sampling locations (a) location 1 (b) location 2 (c) location 3

A total of 17 isolates of cellulolytic bacteria were obtained from the isolation process carried out. These bacterial isolates can be seen in Table 1.

Table 1. Obtained cellulolytic bacterial isolate

Bacterial isolate code	Sample	Sum
S1A 1, SIA 2, S2A, S3B	Freet	4 isolates
AL 3	Water	1 isolate
AP 3, AP 6, AP 8, AP 9, AP 14, AP 17, AP 20, AP 22, AP 24, AP 25, AP 26, AP 27	Mud	12 isolates

These bacterial isolates are then tested phenetic in the form of colony morphology, cell morphology, physiological (biochemical) properties and the influence of environmental factors. The test results are then used as a reference in identifying bacteria.

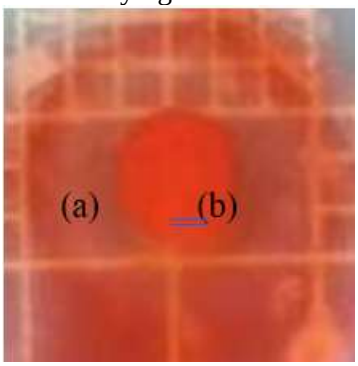

Figure 2. Cellulolytic Index Test (a) clear zone (b) bacterial colonies

The clear zone formed and the bacterial colony are then measured in diameter using a caliper. The data obtained are used to measure the Cellulolytic Index (IS) using a formula according to Choi *et al*.(2005) following.

$$\text{Cellulolytic index} = \frac{DB-DK}{DK} \quad (1)$$

Information:

DB = Clear zone diameter (mm)

DK = Colony diameter (mm)

The calculation results along with the Cellulolytic Index (IS) category are presented in Table 2.

Table 2. Bacterial Cellulolytic Index

Bacterial isolates	Cellulolytic Index (mm)	Category IS
S1A 1	0,387	Low
SIA 2	0,235	Low
S2A	0,035	Low
S3B	0,320	Low
TO 3	0,310	Low
AP 3	0,350	Low
AP 6	0,045	Low
AP 8	0,210	Low
AP 9	0,495	Low
AP 14	0,510	Low
AP 17	0,415	Low
AP 20	0,075	Low
AP 22	0,110	Low
AP 24	0,400	Low

Bacterial isolates	Cellulolytic Index (mm)	Category IS
AP 25	0,250	Low
AP 26	0,085	Low
AP 27	0,385	Low

This Cellulolytic Index (IS) value needs to be further confirmed by conducting quantitative tests on cellulase enzyme activity.

3.2. Cellulase enzyme activity testing

Quantitative test of cellulase enzyme activity is to determine the reduced sugar content produced by the enzyme hydrolysis process against the substat. This test is carried out using the DNS (dinitro salicylic acid) method. Enzyme activity is obtained by enzyme absorbance from DNS test against glucose standard curve equation. Absorbance measurements were carried out using a spectrophotometer with an absorbance of 540 nm. The results of measuring the absorbance of the glucose solution are presented in the curve in Figure 3.

The results of the standard curve show a value of y = 0.0005x+0.0752 with a correlation value ($R^{2)}$ = 0.9891. In this formula, the y value is the absorbance value while the x value is the glucose concentration.

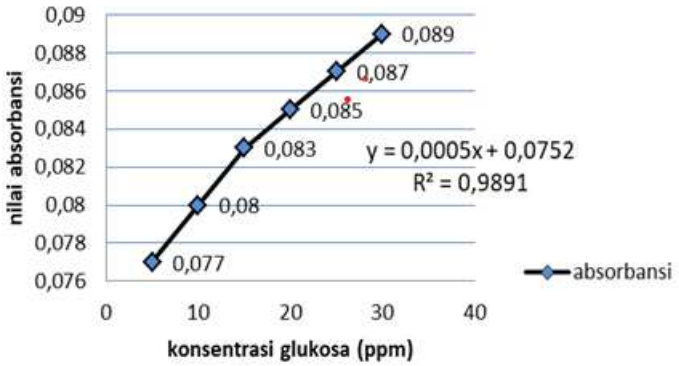

Figure 3. Standard curve of glucose

The glucose concentration obtained is then used to analyze the activity of cellulase enzymes using a formula according to Soeka (2019) according to the following.

$$cellulase\ activity = \frac{(Glucose\ content\ x\ dilution}{(Glucose\ molecule\ weight\ x\ i} \quad (2) \quad \frac{)x1000}{on\ time)}$$

The results of the calculation of cellulase enzyme activity from bacterial isolates obtained are presented in Table 3.

Table 3. Cellulase enzyme activity

Bacterial Isolate	Cellulase Enzyme Activity (U/ml)
AP 14	286,72
AP 3	272,93
AP 9	258,85
S1A 1	239,5
AP 6	223,76
AP 27	217,46
AP 8	217,28
AP 17	207,74
SIA 2	204,31
AP 24	203,85
AP 25	200,33
AP 26	199,59

Bacterial Isolate	Cellulase Enzyme Activity (U/ml)
S2A	198,3
AP 22	196,54
AP 20	187,37
S3B	179,5
TO 3	168,11

The value of enzyme activity indicates the number of micromoles of substrate converted into product per unit time under optimal conditions. This value is generally expressed in enzyme units (U) with 1U = 1 μmol min^{-1} (Seftiono, 2017). So that the higher the value of cellulase enzyme activity indicates the greater the amount of cellulose that can be broken down, which means the cellulase enzyme activity is greater. Based on the results of these calculations, it is known that the highest cellulase enzyme activity in AP 14 bacterial isolate is 286.72 U / ml while the lowest cellulase enzyme activity in AL 3 bacterial isolate is 168.11 U / ml. The size of the activity of this cellulase enzyme affects the levels of reducing sugar (glucose) produced.

3.3. Identification of cellulase-producing bacterial isolates by numerical-phenetic systematic method

The results of testing the phenetic character of all cellulolytic bacterial isolates obtained can be seen in Table 4.

The results of these various tests are then used as a basis for phenetic identification. The collected data is made in binary notation with the number 1 for positive results and the number 0 for negative results. Also added with the phenetic character of the reference strain in the form of *Bacillus pumilus*, *Bacillus stearothermophyllus* and *Streptomyces* sp. The selection of this reference strain is based on the similarity with test isolates based on *Simple Profile Matching* that was done. Using the MVSP 3.1 application, the data is then constructed into the form of a dendogram. The results of the construction of the dendogram are shown in Figure 4.

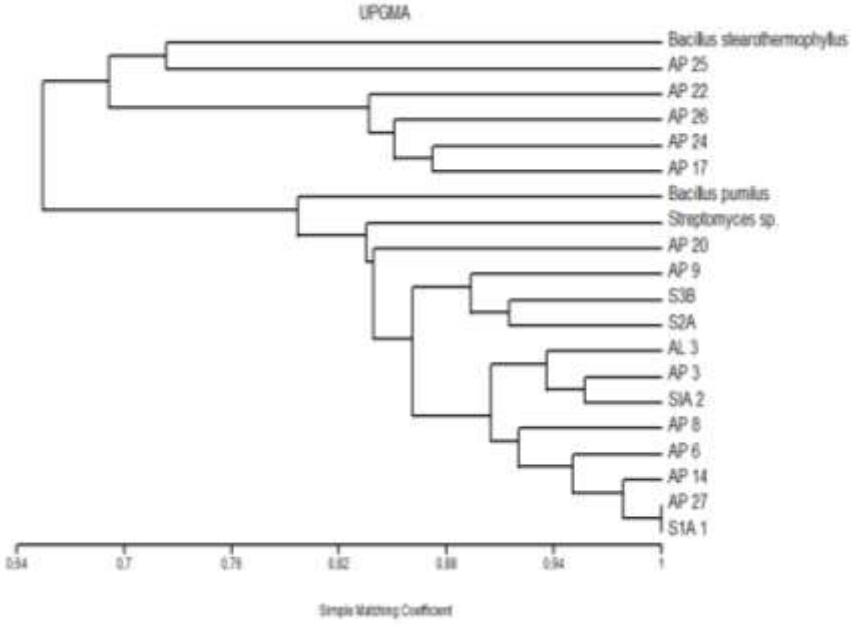

Figure 4. Dendogram of phenetic diversity of cellulolytic bacterial isolates

The dendogram shows the degree of similarity between isolates of test cellulolytic bacteria. The higher the degree of similarity, the greater the kinship of cellulolytic bacterial isolates. The dendogram showed the presence of 19 bacterial clusters. AP 27 and S1A 1 bacterial isolates occupy the same cluster, it is suspected that the bacterial isolate is the same type of bacteria, so further testing needs to be done to ascertain whether the isolate is the same type of isolate or not. While the other 15 bacterial isolates occupying different clusters show different phenetic characters. The degree of

similarity of test bacteria with the reference strain is shown by the similarity index value which can be seen in Table 5.

Table 5. Simple Matching Coefficient similarity matrix

Bacterial isolates	*Bacillus pumilus*	*Bacillus stearothermophyllus*	*Streptomyces* sp.
S1A 1	83%	68%	**89%**
S1A 2	**87%**	72%	85%
S2A	**79%**	72%	77%
S3B	75%	64%	**77%**
TO 3	**81%**	70%	79%
AP 3	**83%**	72%	81%
AP 6	**87%**	64%	85%
AP 8	77%	70%	**87%**
AP 9	77%	62%	**83%**
AP 14	81%	66%	**87%**
AP 17	**72%**	66%	62%
AP 20	72%	57%	**83%**
AP 22	70%	**72%**	51%
AP 24	68%	**75%**	62%
AP 25	57%	**72%**	47%
AP 26	68%	**70%**	62%
AP 27	83%	68%	**89%**

Based on the similarity index value, there are 6 bacterial isolates with a similarity index of ≥ 72% to the reference *Bacillus pumilus.* The bacterial isolates are S1A 2, S2A, AL 3, AP 3, AP 6 and AP 17. There are 4 bacterial isolates with a similarity index of ≥ 70% to *Bacillus stearothermophyllus.* These bacterial isolates are AP 22, AP 24, AP 25, and AP 26. There were 7 bacterial isolates with a similarity index of ≥ 77% against the reference strain *Streptomyces* Sp. The bacterial isolates are S1A 1, S3B, AP 8, AP 9, AP 14, AP 20 and AP 27. These results showed that cellulolytic bacterial isolates that were successfully isolated from the Wana Tirta Kulon Progo Mangrove Forest were dominated by bacterial isolates that had similarities to bacteria *Streptomyces* Sp.

Similar research by Kurniawan, et al (2018) in the Mangrove Forest of Bangka Regency mentioned the discovery of cellulolytic bacteria in the form of *Bacillus alvei* and *Bacillus pumilus.* Research by Vijayakumar and Malatji (2014) mentions the discovery of cellulolytic bacteria in the form of *Streptomyces* sp. in polluted soil. As well as research by Satriani, et al (2019) in the Tarakan Mangrove Forest stated that the identified cellulolytic bacteria isolates were dominated by *Bacillus* sp. Some of these studies show that cellulolytic bacteria that dominate mangrove forests are *Bacillus* sp. In line with this, cellulolytic bacterial isolates that were successfully isolated from the Wana Tirta Kulon Progo Mangrove Forest were also dominated by the genus Bacillus as many as 10 isolates

4. CONCLUSION

Based on the research and discussion conducted, it can be concluded that cellulolytic bacteria can be isolated from the Wana Tirta Kulon Progo mangrove forest. 4 isolates were obtained from litter samples, 1 isolate from water samples and 12 isolates from mud samples. Based on the dendogram of phenetic diversity of cellulolytic bacterial isolates, 19 bacterial clusters were obtained. There are 6 bacterial isolates with a similarity index of ≥ 72% to the reference Bacillus pumilus in the form of isolates S1A 2, S2A, AL 3, AP 3, AP 6 and AP 17. There are 4 bacterial isolates with a similarity index of ≥ 70% to Bacillus stearothermophyllus in the form of AP 22, AP 24, AP 25, and AP 26 isolates. And there are 7 bacterial isolates with a similarity index of ≥ 77% against the reference strain Streptomyces Sp. in the form of isolates S1A 1, S3B, AP 8, AP 9, AP 14, AP 20 and AP 27. The highest cellulase enzyme activity in cellulolytic bacterial isolates from Wana Tirta Kulon Progo Mangrove Forest was found in AP 14 bacterial isolates, which amounted to 286.72 U/ml.

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Table 4. Tabulation of phenetic test results of cellulolytic bacterial isolates

Character phenetic	Cellulolytic bacteria isolate code																
	S1A 1	S1A 2	S2A	S3B	TO 3	AP 3	AP 6	AP 8	AP 9	AP 14	AP 17	AP 20	AP 22	AP 24	AP 25	AP 26	AP 27
Colony form:																	
Irregular	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+
Circular	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-
Colony color:																	
White	+	+	-	-	-	-	+	-	+	+	-	+	-	-	-	-	+
Yellowish white	-	-	+	-	-	-	-	+	-	-	-	-	+	+	-	-	-
White middle yellow	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-
White orange	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Orange	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-
Pink	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Colony elevation:																	
Umbonate	+	+	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+
Flat	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-
Conveks	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Colony edges:																	
Undulate	+	+	-	-	+	+	+	+	-	+	+	-	-	+	-	-	+
Lobate	-	-	+	+	-	-	-	-	+	-	-	-	+	-	-	+	-
Entire	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-
Cell shape:																	
Basil	-	+	+	+	+	+	-	-	+	-	-	-	+	-	+	-	-
Coccus	+	-	-	-	-	-	+	+	-	+	+	+	-	+	-	+	+
Types of grams	+	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	+
Endospora	+	+	+	-	-	+	+	+	-	-	-	+	-	-	-	+	+
Cellulose hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Amylum hydrolysis	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Oxygen requirement:																	
Facultative anaerobic	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
aerobic	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Hidrolisa gelatin	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucose fermentation	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	+
Sucrose fermentation	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	+
Lactose fermentation	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	+

Catalse test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate test	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Indole test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H2S production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Motility test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
VP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PH 5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PH 7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PH 8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mouth 4ºC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mouth 32ºC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mouth 80ºC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-