ISOLATION, CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF DYE DECOLORIZING BACTERIA FROM TEXTILE SLUDGE

By

Sumaiya Sultana
15226004

A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of
Bachelor in Microbiology

Department of Mathematics and Natural Sciences
Brac University
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Declaration

It is hereby declared that

1. The thesis submitted is my/our own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
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Student's Full Name & Signature:

Sumaiya Sultana

15226004

Student Full Name
Student ID

Approval

The thesis titled "Isolation, Characterization and Phylogenetic Analysis of Dye Decolorizing Bacteria from Textile Sludge" submitted by Sumaiya Sultana (15226004) of Spring, 2020 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor in Microbiology on 27th February, 2020.

Examining Committee:

Supervisor:
(Member)

_____
Romana Siddique
Senior Lecturer, Department of Mathematics and Natural Sciences,
Brac University

Program Coordinator:
(Member)

_____
Mahbubul Hasan Siddiqee
Senior Lecturer, Department of Mathematics and Natural Sciences,
Brac University

Departmental Head:
(Chair)

_____
Dr. A F M Yusuf Haider
Professor and Chairperson, Department of Mathematics and Natural Sciences,
Brac University

Abstract

Textile industries use various dyes which have become one of the key sources of environment pollution. As a result, it is necessary to remove these undesirable dyes from the effluent that is released to the environment. This study was envisaged to obtain potential bacterium that can degrade the reactive dyes present in soil/sludge samples collected from the textile disposal sites. Samples were collected aseptically from Narsingdi, Bangladesh. On the basis of the degradation rate, three bacterial strains were isolated using salt media (SM) for two dyes named Setazol Blue BB and Black B. Isolated bacterial strains were identified as *Bacillus* sp. (SSR_S-4_19, SSR_B-3_19), *Pseudomonas* sp. (SSR_S-5_19) by 16srRNA gene sequencing analysis. For the decolorization experiment, SM broth containing specific dyes (1%) were inoculated with each of the strains. All the three strains could decolorize the soil sample within 24h which was estimated using spectrophotometry. The decolorization rate for Setazol Blue BB was 87.7% and 86.5% in 24hr for the strains SSR_S-4_19 and SSR_S-5_19 respectively. On the other hand, for Black B it was 92.6% and 85.22% in 24hr for the strains SSR_B-3_19 and SSR_S-4_19 respectively. Since these strains have been efficient in decolorizing the dyes, in future it may be a promising strain for industrial use.

Keywords: reactive dye; dye degrading bacteria; textile effluent; decolorization rate; salt media (SM); spectrophotometry

Dedication

This is dedicated to my family for their constant support, love and encouragement in completing this project.

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My heartiest gratitude is for my parents who helped me collecting the samples and dyes which were the most important thing for this project. Besides, I would like to thank my friend Mridul who also helped me in collecting some textile dyes.

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List of Acronyms

SM	Salt media
rPm	Rotation per minute
NA	Nutrient agar
min	minute
ml	Milliliter
g/l	Gram per liter
hr	hour
SD	Standard deviation
LB	Luria-Bertani medium
MSA	Mannitol salt agar
MAC	MacConkey agar
XLD	Xylose lysine deoxycholate
EMB	Eosin methylene blue
BCA	Bacillus Agar
MIU	Motility indole urease
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
BLAST	Basic Local Alignment Search Tool
UPGMA	Unweighted Pair Group Method with Arithmetic Mean

Chapter 1

Introduction

Textile industry plays a major role in the economy of a country but at the same time it has become one of the key sources of environment pollution(Ashfaq Ahmad, 2014). Textile industry requires large volume of water for their dyeing operation and around 90% of this is released as wastewater(Dipankar Chandra Roy, Sudhangshu Kumar Biswas, Ananda Kumar Saha, Biswanath Sikdar, Mizanur Rahman, Apurba Kumar Roy, Zakaria Hossain Prodhan, 2018). This wastewater contains various dyestuffs which when released in the environment causes water pollution(Barathi Selvaraj, Chinnannan Karthik, Nadanasabapathi S, 2020). In addition, humans and animals are at a higher risk of diseases and infections such as cancer, skin allergy due to this untreated release of textile effluent in the water bodies(S.Barathi, 2015)(K.R. Mahbub, A. Mohammad, M.M. Ahmed, 2012). This untreated or inappropriate discharge of textile effluent into the environment decreases penetration of sunlight which results in reduction of photosynthetic activity leading to toxic effects on marine system and dissolved oxygen concentration(Dipankar Chandra Roy, Sudhangshu Kumar Biswas, Ananda Kumar Saha, Biswanath Sikdar, Mizanur Rahman, Apurba Kumar Roy, Zakaria Hossain Prodhan, 2018)(Mohamed El Bouraie, 2016). Moreover, wastewater released from dyeing industry also has adverse effect on total organic carbon, biological oxygen demand and chemical oxygen demand(Maulin P Shah, Kavita A Patel, Sunu S Nair & Industrial, 2013). Hence, it is obligatory to develop a method for the removal of toxic dye from the textile effluent which is effective, economical and ecofriendly(Barathi Selvaraj, Chinnannan Karthik, Nadanasabapathi S, 2020).

Numerous physicochemical methods have been used for the removal of dyes from the textile effluent including adsorption, irradiation, ion exchange, oxidative process, ozonation,

coagulation(Sanmuga Priya Ekambaram, Senthamil Selvan Perumal, 2016). However, these methods are expensive, ineffective and sometimes causes secondary pollution(N. Sriram, 2015)(R.G. Saratale, G.D. Saratale, J.S. Chang, 2011). Thus, a cheaper yet effective and environment friendly methods are in need. In recent decades, biological methods have been examined like biodegradation and biosorption by microbial biomass which may take place in an aerobic, anaerobic or mixed treatment processes with bacteria, fungi, yeasts and algae(Md. Ekramul Karim, Kartik Dhar, 2018)(Yuanyuan Qu, Shengnan Shi , Fang Ma, 2010).

Microbiological treatment is comparatively cheaper, environment friendly and more efficient than the conventional method(S. Menaka and S. Rana, 2016)(Bella Devassy Tony, Dinesh Goyal, 2009). In addition to that, microorganisms are capable of completely mineralizing textile dyes under optimal conditions(Ken Meerbergen, Kris A. Willems, Raf Dewil, Jan Van Impe, Lise Appels, 2018)(Rodrigues de Almeida et al., 2019). In recent years, single bacterial strain has been identified having the ability to degrade textile reactive dyes with high efficiency. On the contrary, pure culture have advantages over mixed culture such as having the knowledge about the degradation pathway with prediction that the degradation end product is nontoxic(A. Karthikeyan, 2013). Thus, biological method is considered as best treatment method for textile dye degradation. Bacteria that are able to decolorize the textile dye can be isolated from various abode inclusive of soil, water, colored effluents, human/animal excreta, and contaminated food materials(Ken Meerbergen, Kris A. Willems, Raf Dewil, Jan Van Impe, Lise Appels, 2018).

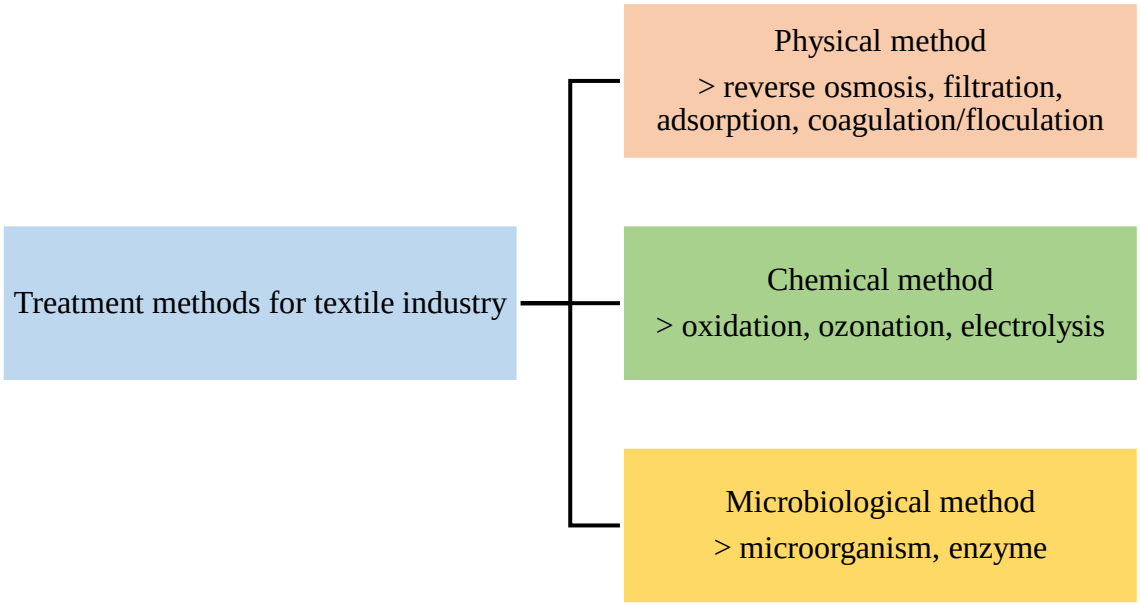


Figure 1: Types of treatment method

The objective of this study was to isolate an efficient bacterium that can fully decolorize the textile effluent containing dyes. In this study, bacteria were isolated from textile effluent which was collected from effluent discharge site of Madhabdi, Narsingdi. Initially five reactive dyes were used namely Setazol Blue BB, Agrazol Red 3BS, Agrazol Yellow 3RS, Orange P2R and Black B within which two dyes were completely decolorized. Dye decolorization was performed under aerophilic condition till no visual color was detected(Franciscon Elisangela, Zille Andrea, Dias Guimaro Fabio, Ragagnin de Menezes Cristiano, Durrant Lucia Regina, 2009). Finally, the decolorization rate was measured using spectrophotometer.

Chapter 2

Materials and methodology

2.1 Sample collection

Textile effluent sample was collected aseptically from Narsingdi, Bangladesh. After collection, sample was immediately transported to lab and kept in 4˚C.

2.2 Chemicals, dyes and media

Dyes used for this study were Setazol Blue BB, Agrazol Red 3BS, Agrazol Yellow 3RS, Orange P2R, Turquoise Blue G and Black B which were collected from Shah Amanat Knitting & Dyeing Industries Ltd. and Bengal Hurricane Dyeing & Printing Pvt. Ltd., Bangladesh. Stock solution of the dyes were prepared by dissolving 1g of each dye into 100ml of sterile distilled water(Md. Ekramul Karim, Kartik Dhar, 2018). For this study, SM broth was used which contained: peptone 10g/l; KH_2PO_4 1.9g/l; K_2HPO_4 0.6g/l; NH_4Cl 1g/l; Yeast extract 1g/l.

2.3 Isolation, identification and screening of dye decolorizing bacteria

Sample was initially inoculated in SM broth containing dye. After visual decolorization, sample from this container was diluted up to 10^{-7} and plated on NA plate(Walaa A. Al-Shareef, Salwa S.I. Afifi, Mohamed A. Ramadan, 2018). From this NA plate, 24 colonies were randomly selected on the basis of colony morphology(Farhana Hussain, Md. Reazul Karim, Fahmida Hossain, 2018) and tested for decolorization of five reactive dyes in shaking condition(80rpm) at 37˚C(Md. Zobaidul Hossen, Md. Eleus Hussain, Al Hakim, Kamrul Islam, Md. Nizam Uddin, 2019)(Manjinder Singh Khehra, Harvinder Singh Saini, Deepak Kumar Sharma, Bhupinder Singh Chadha, 2005). Isolates that degrade one of the six dyes within 48hr were selected for further study.

2.4 Decolorization assay

Experiment was conducted in 100ml conical flask under shaking (80rpm, 37˚C) condition. Samples from the decolorized container was centrifuged at 10,000rpm for 15min at 4ºC(Manjinder Singh Khehra, Harvinder Singh Saini, Deepak Kumar Sharma, Bhupinder Singh Chadha, 2005). Then clear supernatant was taken for the analysis using spectrophotometry. All assays were performed triplicate and compared with uninoculated controls. Absorbance was measured at 540-600nm wavelengths(M. Ponraj, K. Gokila, 2011)(Naeimeh Enayatizamir, Fatemeh Tabandeh, Susana Rodríguez-Couto, Bagher Yakhchali, Hossein A. Alikhani, 2011)(Guang Guo, Xiaohua Li, Fang Tian, Tingfeng Liu, Feng Yang, Keqiang Ding, Chong Liu, Jiasheng Chen, 2020) and there was peak at 600nm(Chandrakant R. Holkara, Harshit Arora, Dibyadeep Halder, 2018)(Carolina Heyse Niebisch, Alexandre Knoll Malinowski, Ruth Schadeck, David A. Mitchell, Vanessa Kava-Cordeiro, 2010). Absorbance was taken in every 24hr. Absorbance was taken using Thermo Scientific™ GENESYS™ 10S UV-Vis Spectrophotometer.

The decolorization assay of different isolates were expressed as per the following Eq.(Cai Feng, Chen Fang-yan, 2014)(M. Ponraj, K. Gokila, 2011)(Wycliffe C.Wanyonyi, John M.Onyari, Paul M.Shiundu, 2019)(Chandrakant R. Holkara, Harshit Arora, Dibyadeep Halder, 2018)

$$\text{Decolorization Rate} = \frac{A-B}{A} \times 100$$

A= initial absorbance of dye solution containing sample

B= final absorbance of dye solution containing sample

2.5 Biochemical tests of dye decolorizing bacteria

For the presumptive identification of the characteristics of selected isolates, different biochemical tests were performed including gram's staining, triple sugar iron test, indole test, methyl red test, Voges–Proskauer test, catalase test, oxidase test, starch hydrolysis test, lipid hydrolysis test, casein hydrolysis test, nitrate reduction test, blood agar test, motility indole urease test and some tests on selective media like MSA, MAC, XLD, EMB, BCA.

2.6 DNA extraction, PCR, gel electrophoresis

DNA extraction of the selected strains were performed using Wizard® Genomic DNA Purification Kit. After successful DNA extraction, PCR was performed using two sets of universal bacterial 16SrRNA gene primers. Primer sets that were used include fD1; rp2 and 27f; 1492r. This PCR was set up for 35 cycles following hot start PCR protocol.

Primer sequence for primer-1 were fD1 (5' AGAGTTTGATCCTGGCTCAG 3') and rP2 (5' ACGGCTACCTTGTTACGACTT 3')(C. Ota-Tsuzuki, A.T.P. Brunheira, 2008)(T Gotoh, H Noda, 2007)(Heekyung Chung, Myoungsu Park, Munusamy Madhaiyan, Sundaram Seshadri, Jaekyeong Song, Hyunsuk Cho, 2005). On the other hand, sequence for primer-2 were 27f (5' AGAGTTTGATCMTGGCTCAG 3') and 1492r (5' TACGGYTACCTTGTTACGACTT 3')(Ram Chandra, Anuradha Ghosh, Rakesh Kumar Jain, 2006). In addition to that, gel electrophoresis was also performed in 1% gel to ensure that DNA extraction was successful and no primer-dimer was formed in PCR.

2.7 PCR purification and 16srRNA gene sequencing

PCR product was purified using Wizard® PCR purification kit. For the 16srRNA gene sequencing, the purified samples were sent to Invent technologies, Bangladesh.

2.8 Multiple sequence alignment and phylogenetic tree analysis

After having the sequences, it was analyzed at NCBI server using BLAST (blastn) tool and matching sequences from hit list was downloaded in FASTA format(Safia Moosvi, Xama Kher, 2007). Besides, multiple sequences were aligned using MEGA X software. Along with that, phylogenetic tree was also constructed by Neighbour-joining, UPGMA, Maximum likelihood method, Maximum evolution.

Chapter 3

Result and discussion

3.1 Isolation, identification and screening of dye degrading bacteria

Initially, six textile reactive dyes were used to observe the decolorization activity. For the dyes Agrazol Red 3BS, Agrazol Yellow 3RS, Orange P2R and Turquoise Blue G there were no observable decolorization. On the other hand, for Setazol Blue BB and Black B there were observable decolorization thus absorbance was measured and decolorization rate was measured with the mentioned equation.

$$\mathrm{OD}_{600} = \frac{\mathrm{A}_{\mathrm{initial}} - \mathrm{A}_{\mathrm{final}}}{\mathrm{A}_{\mathrm{initial}}}$$

From the decolorized flasks, bacteria were isolated using spread plate method and randomly 24 colonies were selected for further study. After that, these 24 colonies were inoculated into SM broth containing Setazol Blue BB and Black B dyes. Each of the 24 colonies were enriched using LB media in shaker incubator at 37˚C, 150rpm for 24hr(M. Dexilin, V. Elavarasi, 2013). After enrichment, 1ml of each enriched culture were inoculated into SM broth containing each dye. Then the flasks were kept in shaker incubator at 37˚C, 80rpm and absorbance was taken in 24hr time interval.

Initial absorbance (0hr) and following observable decolorization final absorbance (24hr) was taken using spectrophotometer at 600nm. For each of the dye, only the decolorized flask was taken and further experimented. Among the 24 colonies only 3 colonies were found to be effective in decolorizing.

3.2 Decolorization assay

After final experiment, 3 isolates that were found to be efficient in decolorizing named SSR_S-4_19, SSR_S-5_19 and SSR_B-3_19 where SSR_S-4_19, SSR_S-5_19 decolorized Setazol Blue BB and SSR_S-4_19, SSR_B-3_19 decolorized Black B. Initial decolorization rate of Setazol Blue BB from sludge sample was 91.14% and for Black B it was 96.08%. Isolates SSR_S-4_19, SSR_S-5_19 and SSR_B-3_19 was inoculated in the media containing dye and observed for decolorization activity. However, the decolorization activity was visualized within 24hr of inoculation. As per the OD, the decolorization rate for Black B were 85.22% and 92.6% by SSR_S-4_19 and SSR_B-3_19 respectively. On the other hand, decolorization rate for Setazol Blue BB were 87.7% and 86.5% b SSR_S-4_19 and SSR_S-5_19 respectively.

3.2.1 Decolorization of Setazol Blue BB

Within 24hr efficient decolorization was observed for Setazol Blue BB. The average decolorization rate was 91% within 24hr.

Control *sample inoculated* *decolorization*

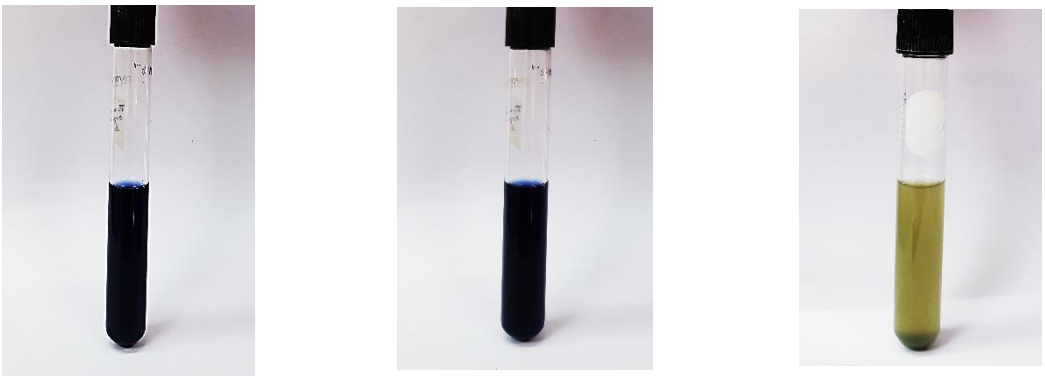
Figure 2: Decolorization of Setazol Blue BB

Name	Control	0hr	24hr	Decolorization rate
Average absorbance	0.945	1.005	0.089	91.14%
Average SD of SSR_S-4_19	0	0.00163	0.00124	N/A
Average SD of SSR_S-5_19	0	0.0357	0.00623	N/A

Table 1: Average absorbance & SD of Setazol Blue BB

3.2.2 Decolorization of Black B

For Black B, the decolorization was observed within 16hr but the absorbance was measured in 24hr interval. Average decolorization of this dye was 96% in 24hr.



Control *inoculated* *decolorization*

Figure 3: Decolorization of Black B

Name	Control	0hr	24hr	Decolorization rate
Average absorbance	1.406	1.48	0.058	96.08%
Average SD of SSR_B-3_19	0	0.1322	0.00216	N/A
Average SD of SSR_S-4_19	0	0.0208	0.0463	N/A

Table 2: Average absorbance & SD of Black B

3.2.3 Decolorization by specific isolates SSR_S-4_19, SSR_S-5_19 and SSR_B-3_19

Among the isolates, SSR_S-4_19 was able to decolorize both of the two dyes efficiently whereas SSR_S-5_19 and SSR_B-3_19 was able to decolorize Setazol Blue BB and Black B respectively. For each of the isolates, triplets were done and the average were taken for the illustration. Moreover, standard deviation of the samples from the control were also measured and graphically represented.

Name of dye	Isolate	control	0hr	24hr	Decolorization rate
Black B	SSR_S-4_19	1.506	1.589	0.235	85.22%
	SSR_B-3_19	1.506	1.633	0.119	92.6%

Table 3: Decolorization of Black B by SSR_S-4_19 & SSR_B-3_19

Name of dye	Isolate	control	0hr	24hr	Decolorization rate
Setazol Blue BB	SSR_S-4_19	0.945	0.977	0.119	87.7%
	SSR_S-5_19	0.945	0.959	0.129	86.5%

Table 4: Decolorization of Setazol Blue BB by SSR_S-4_19 & SSR_S-5_19

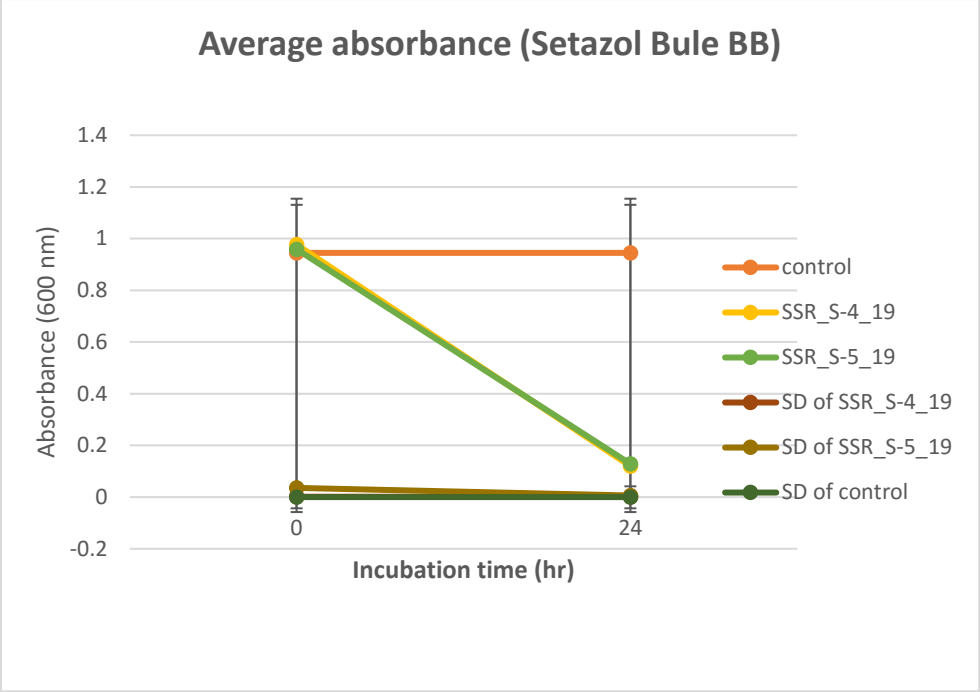


Figure 4: Average absorbance & SD of Setazol Blue BB

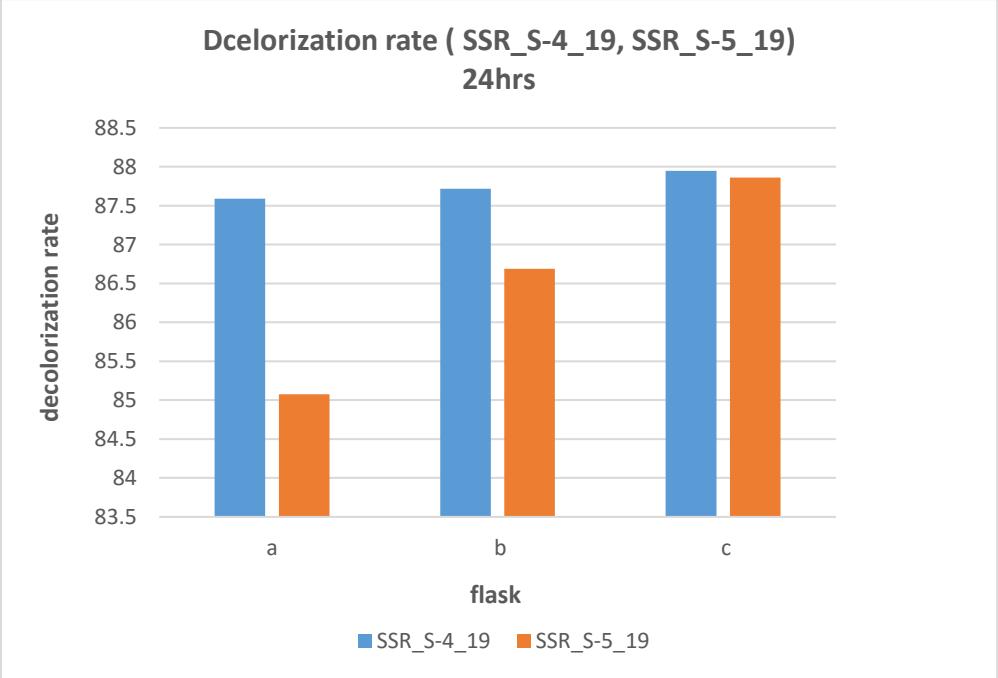


Figure 5: Decolorization of Setazol Blue BB by SSR_S-4_19, SSR_S-5_19

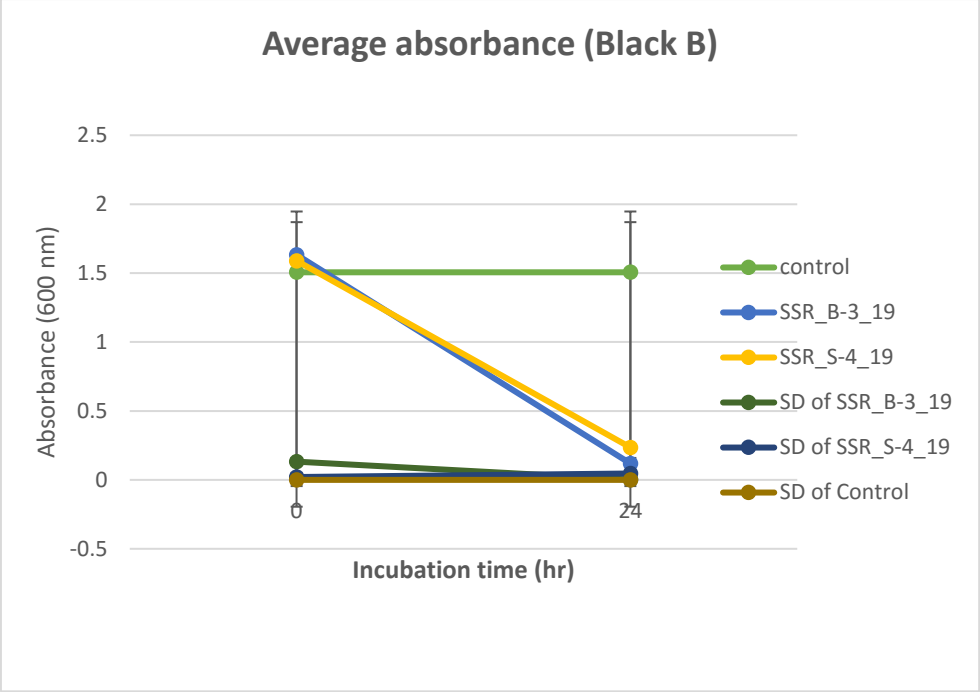


Figure 6: Average absorbance & SD of Black B

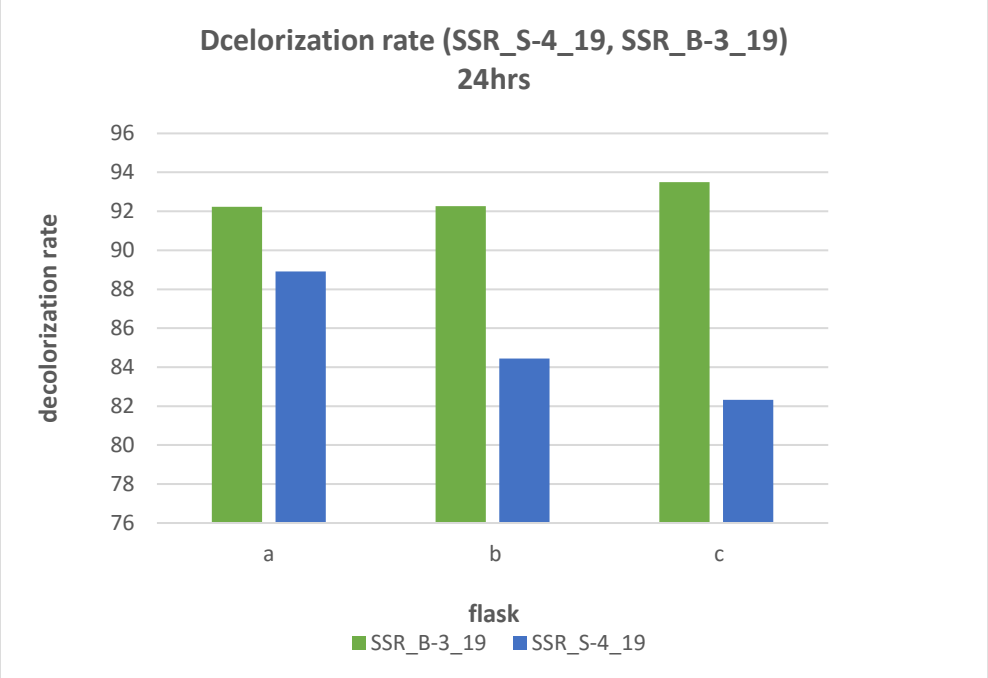


Figure 7: Decolorization of Black B by SSR_S-4_19, SSR_B-3_19

3.3 Biochemical tests of dye decolorizing bacteria

Firstly, gram staining was done to identify whether the bacteria was gram positive or negative. According to the staining result, SSR_B-3_19 and SSR_S-4_19 were gram positive whereas SSR_S-5_19 was gram negative bacteria(Sandle, 2004). Secondly, triple sugar iron was done to identify whether the isolates had the ability to ferment glucose, lactose and sucrose(Lehman, 2013). In TSI test, SSR_B-3_19 and SSR_S-4_19 showed positive result and SSR_S-5_19 showed negative test result. Moreover, to identify the isolates indole test, methyl red, Voges–Proskauer test, citrate utilization test, catalase test, oxidase test, starch hydrolysis, lipid hydrolysis, casein hydrolysis test was done where only SSR_S-5_19 was positive for citrate utilization test, catalase test and starch hydrolysis test. On the other hand, SSR_B-3_19 and SSR_S-4_19 gave positive result for nitrate reduction, BCA and negative result for β-hemolysis, MSA. In addition, SSR_S-5_19 gave positive result for MAC, XLD, EMB and MIU whereas SSR_B-3_19 and SSR_S-4_19 gave negative result for MIU.

SL No.	Tests name	SSR_B-3_19	SSR_S-4_19	SSR_S-5_19
1	Gram's staining	+	+	-
2	Triple sugar iron	+	+	-
3	Indole test	-	-	-
4	Methyl red	-	-	-
5	Voges–Proskauer	-	-	-
6	Citrate utilization	-	-	+
7	Catalase	-	-	+
8	Oxidase	-	-	-
9	Starch hydrolysis	-	-	+

10	Lipid hydrolysis	-	-	-
11	Casein hydrolysis	-	-	-
12	Nitrate reduction	+	+	N/A
13	β- hemolysis	-	-	N/A
14	MSA	-	-	N/A
15	MAC	N/A	N/A	+
16	XLD	N/A	N/A	+
17	EMB	N/A	N/A	+
18	BCA	+	+	N/A
19	MIU	-	-	+

Table 5: Biochemical test result

Isolates	**Shape**	**Color**	**Elevation**	**Margin**	**Surface**	**Morphology**
SSR_B-3_19	Irregular	Cream	Raised	Entire	Smooth	Rod
SSR_S-4_19	Irregular	Cream	Raised	Entire	Smooth	Rod
SSR_S-5_19	Irregular	Cream	Flat	Irregular	Smooth	Short rod

Table 6: Colony morphology of isolates

3.4 DNA extraction, PCR, gel electrophoresis

For the further analysis of the isolates, DNA extraction was done using Wizard® Genomic DNA Purification Kit. After that, the extracted DNA samples were amplified through hot start PCR using two universals primer set. In addition, agarose gel electrophoresis was also performed for the conformation of successful DNA extraction. DNA extraction for all the isolates were successful as per agarose gel electrophoresis result. For this experiment, 1% gel was used and the result was observed under UV light.

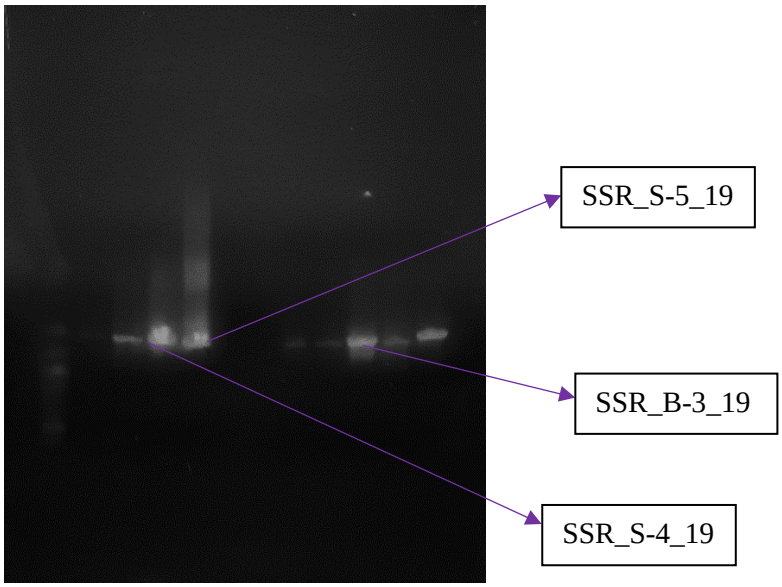


Figure 8: Agarose gel electrophoresis

3.5 PCR purification and 16srRNA analysis

For the analysis of 16srRNA, the PCR samples were purified using the purification kit and sent for 16srRNA sequencing. Based on the sequencing test result the three isolates SSR_B-3_19, SSR_S-4_19, SSR_S-5_19 was identified as *Bacillus* sp., *Bacillus* sp. and *Pseudomonas* sp. respectively. Furthermore, sequences were compared with the available sequences in Genbank using blastn and multiple sequences were aligned(Jing-Long Han, I-Son Ng, Yanni Wang, Xuesong Zheng, Wen-Ming Chen, Chung-Chuan Hsueh, Shi-Qi Liu, 2012). Multiple

sequences were aligned using MEGA X. Moreover, phylogenetic tree was also constructed to analyze the evolutionary relationship of the isolates.

3.5.1 Multiple sequence analysis and phylogenetic tree

For the analysis of MSA, sequences from the blast hit list were downloaded in FASTA format. After that, using this multiple sequence alignment phylogenetic tree was constructed.

SL No.	Name	E-value	Percentage of similarity	Accession number
1	Bacillus andreesenii strain 8-4-E13 16S ribosomal RNA, partial sequence	0	97.95%	NR_125565.1
2	Bacillus sp. Marseille-P3606 partial 16S rRNA gene, strain Marseille-P3606	0	94.18%	LT722675.1
3	Bacillus casamancensis strain TN3 16S ribosomal RNA gene, partial sequence	0	94.18%	JQ415976.1
4	Bacillus sp. mixed culture X11-59 16S ribosomal RNA gene, partial sequence	0	93.95%	KR029396.1
5	Bacillus timonensis strain LNHL41 16S ribosomal RNA gene, partial sequence	0	93.96%	MG008674.1
6	Bacillus sp. KSM-P358 gene for 16S rRNA	0	93.73%	AB073167.1
7	Bacillus humi strain CRRI-HN-6 16S ribosomal RNA gene, partial sequence	0	93.82%	JQ695933.1

8	Bacillus licheniformis strain FA-4 16S ribosomal RNA gene, partial sequence	0	93.49%	MG818961.1
9	Bacillus sonorensis partial 16S rRNA gene, strain Marseille-P3463	0	93.49%	LT714152.1
10	Bacillus salidurans strain yy08 16S ribosomal RNA gene, partial sequence	0	93.50%	MN177179.1
11	Bacillus ciccensis strain H1 16S ribosomal RNA gene, partial sequence	0	94.98%	MK256795.1
12	Bacillus sp. strain FJAT-22122 16S ribosomal RNA gene, partial sequence	0	92.52%	KY949533.1

Table 7: BLAST hit list for SSR_B-3_19

SL No.	Name	E-value	Percentage of similarity	Accession number
1	Bacillus andreesenii strain 8-4-E13 16S ribosomal RNA, partial sequence	0	98.31%	NR_125565.1
2	Bacillus sp. Marseille-P2639 partial 16S rRNA gene, strain Marseille-P2639	0	94.50%	LT598571.1
3	Bacterium strain BS0993 16S ribosomal RNA gene, partial sequence	0	94.33%	MK824181.1
4	Bacillus aerius strain BAB-2472 16S ribosomal RNA gene, partial sequence	0	94.25%	KC443076.1

5	Bacillus beringensis strain BR035 16S ribosomal RNA, partial sequence	0	94.18%	NR_116849.1
6	Bacillus paralicheniformis strain JO-2 16S ribosomal RNA gene, partial sequence	0	94.07%	MF321822.1
7	Bacillus sonorensis strain S3 16S ribosomal RNA gene, partial sequence	0	94.16%	MN056006.1
8	Bacillus humi strain CRRI-HN-6 16S ribosomal RNA gene, partial sequence	0	93.41%	JQ695933.1
9	Bacillus timonensis strain 10403023 16S ribosomal RNA, partial sequence	0	94.08%	NR_133024.1
10	Bacillus licheniformis strain G 16S ribosomal RNA gene, partial sequence	0	94.17%	AY672764.1
11	Bacillus sp. 3458BRRJ 16S ribosomal RNA gene, partial sequence	0	93.91%	FJ215796.2

Table 8: BLAST hit list for SSR_S-4_19

SL No.	Name	E-value	Percentage of similarity	Accession number
1	Pseudomonas mendocina strain CGS7 16S ribosomal RNA gene, partial sequence	0	92.74%	KF886277.1
2	Pseudomonas oleovorans JCM 13981 gene for 16S rRNA, partial sequence	0	92.65%	LC508007.1
3	Pseudomonas plecoglossicida JCM 13969 gene for 16S rRNA, partial sequence	0	92.65%	LC507997.1

4	Pseudomonas pseudoalcaligenes strain KJ1WB 16S ribosomal RNA gene, partial sequence	0	92.66%	MF470189.1
5	Pseudomonas nitroreducens strain EAPn13 16S ribosomal RNA gene, partial sequence	0	92.74%	JF911369.1
6	Gamma proteobacterium JAUIB78 16S ribosomal RNA gene, complete sequence	0	94.92%	DQ983422.1
7	Pseudomonas sp. RM12W 16S ribosomal RNA gene, partial sequence	0	92.32%	EF675623.1
8	Pseudomonas sp. DF7 16S ribosomal RNA gene, partial sequence	0	95.53%	KC294058.1
9	Pseudomonas sp. AD55 16S ribosomal RNA gene, partial sequence	0	92.77%	JQ994361.1
10	Pseudomonas sp. RD_AZIDI_09 16S ribosomal RNA gene, partial sequence	0	92.57%	KU597531.1
11	Pseudomonas nitroreducens subsp. thermotolerans gene for 16S rRNA, partial cds, strain: NBRC 102205	0	92.57%	AB681730.1

Table 9: BLAST hit list for SSR_S-5_19

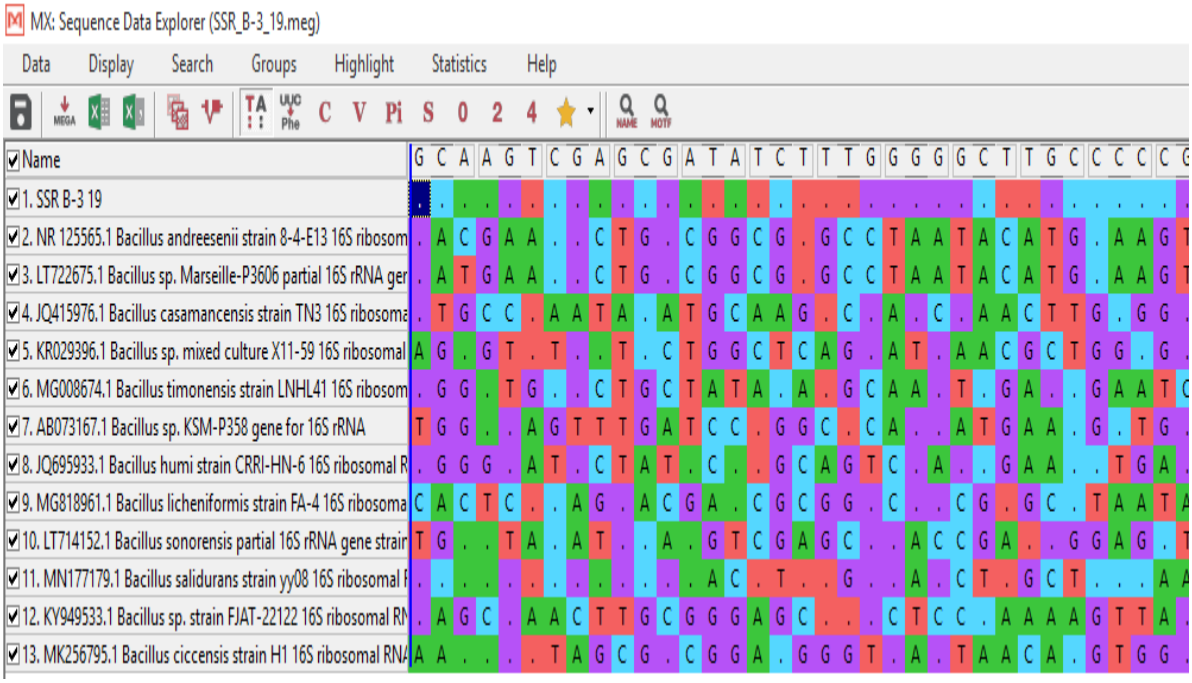


Figure 9: MSA for SSR_B-3_19

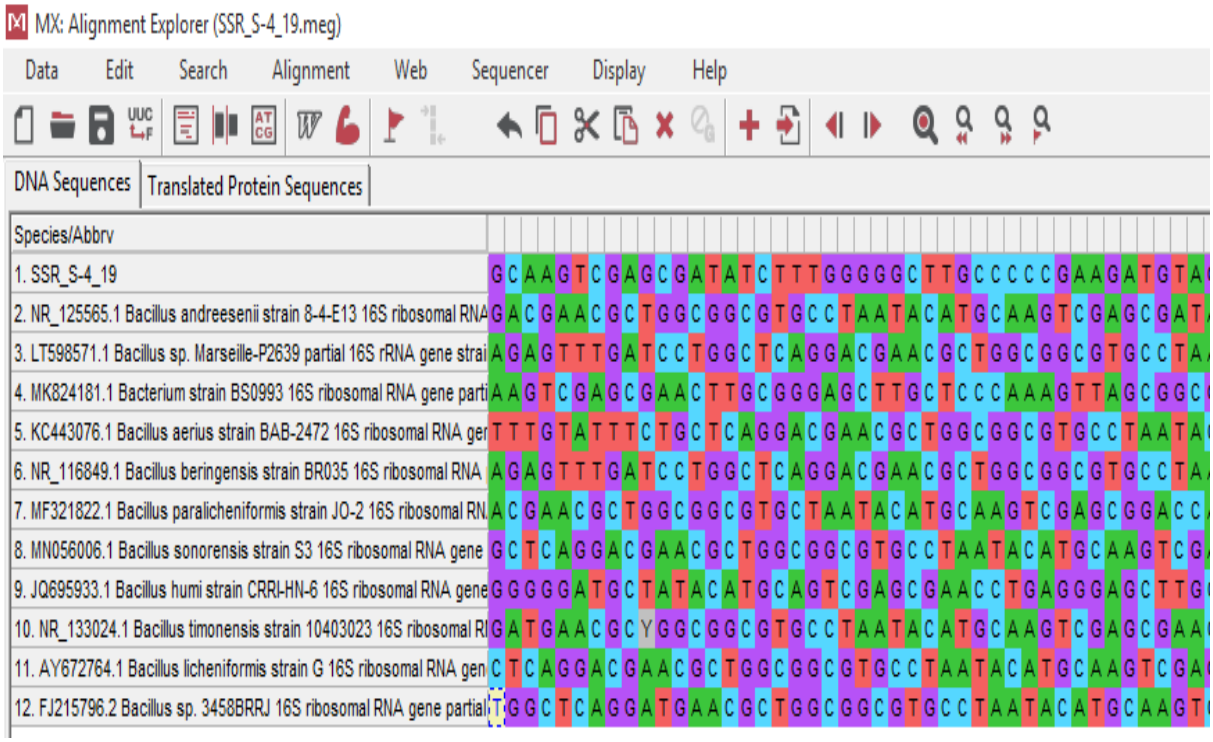


Figure 10: MSA for SSR_S-4_19

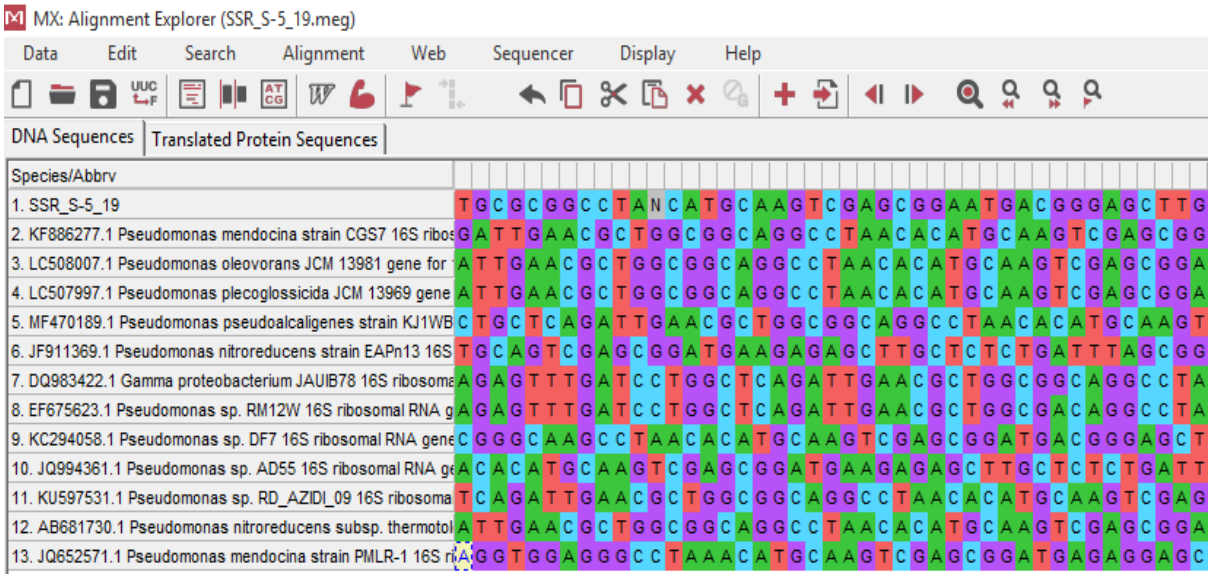


Figure 11: MSA for SSR_S-5_19

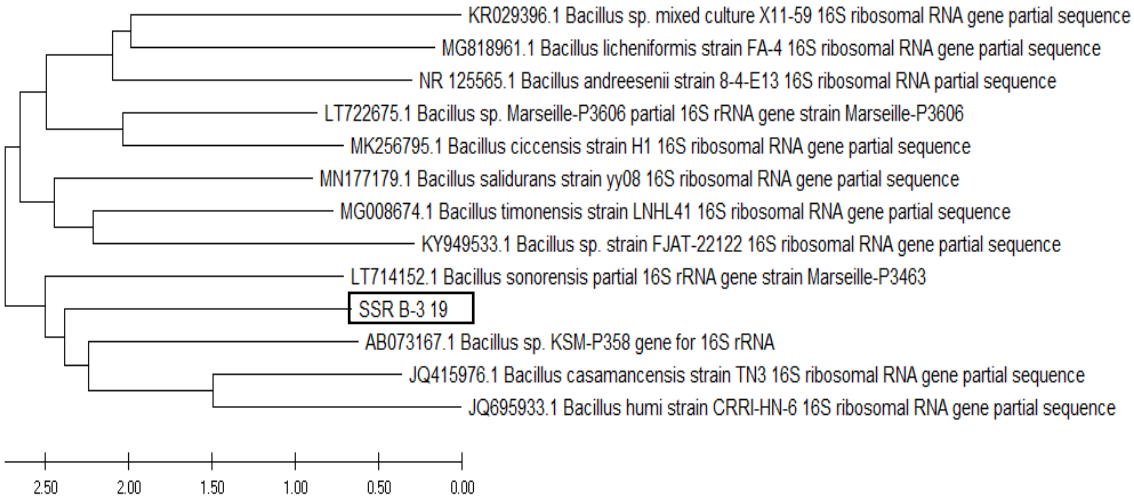


Figure 12: Evolutionary analysis by Maximum evolution (SSR_B-3_19)

The evolutionary history was inferred using the Minimum Evolution method. The optimal tree with the sum of branch length = 23.73405773 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm at a search level of 1. The Neighbor-joining algorithm was used to generate the initial tree. This analysis involved 13 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1548 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

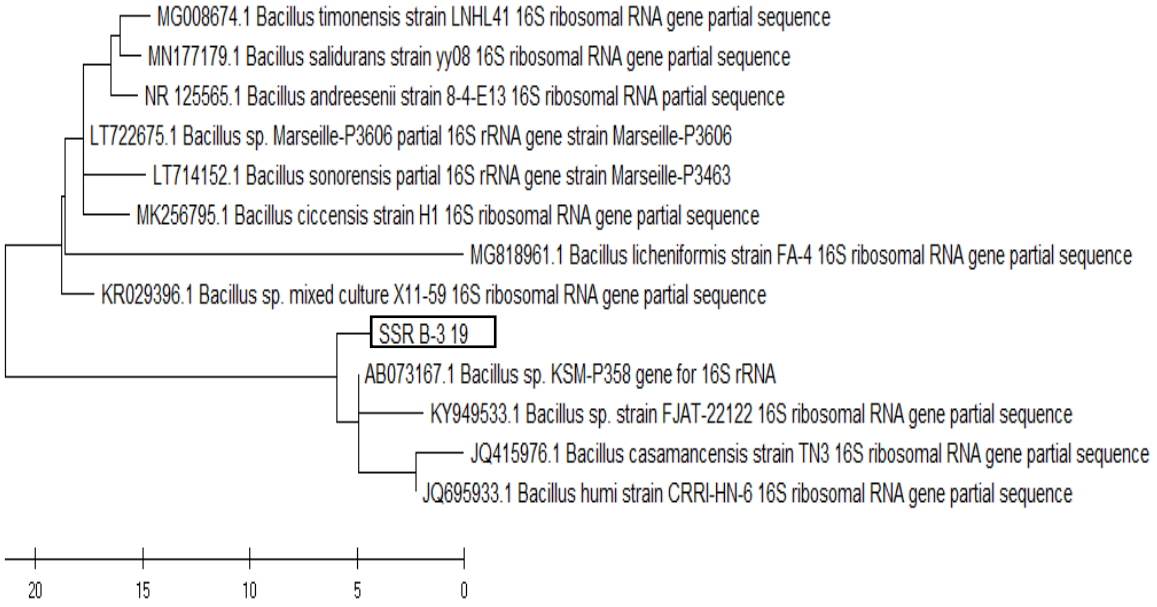


Figure 13: Evolutionary analysis by Maximum likelihood method (SSR_B-3_19)

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood (-23960.16) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 13 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1548 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

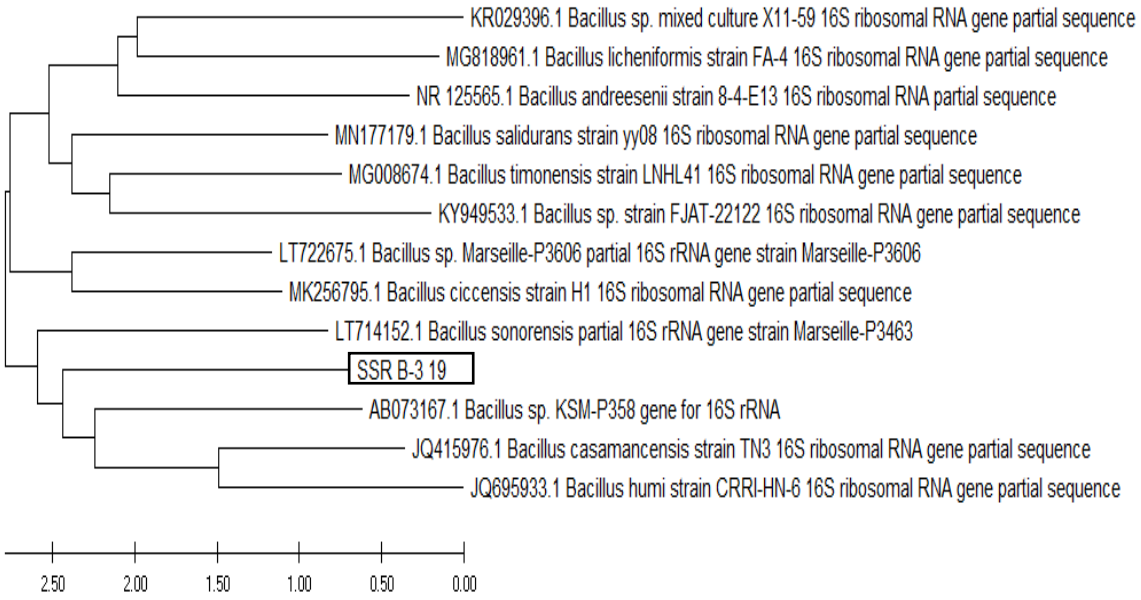


Figure 14: Evolutionary analysis by Neighbour-joining (SSR_B-3_19)

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 23.65283250 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 13 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1548 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

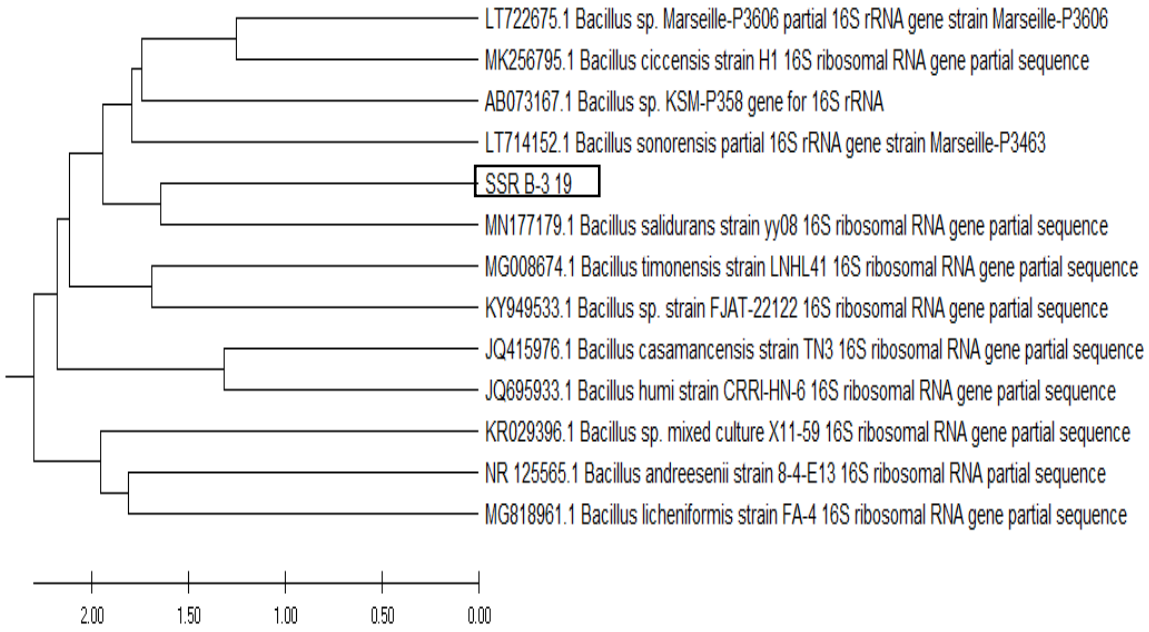


Figure 15: Evolutionary analysis by UPGMA (SSR_B-3_19)

The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 24.01963082 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 13 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1548 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

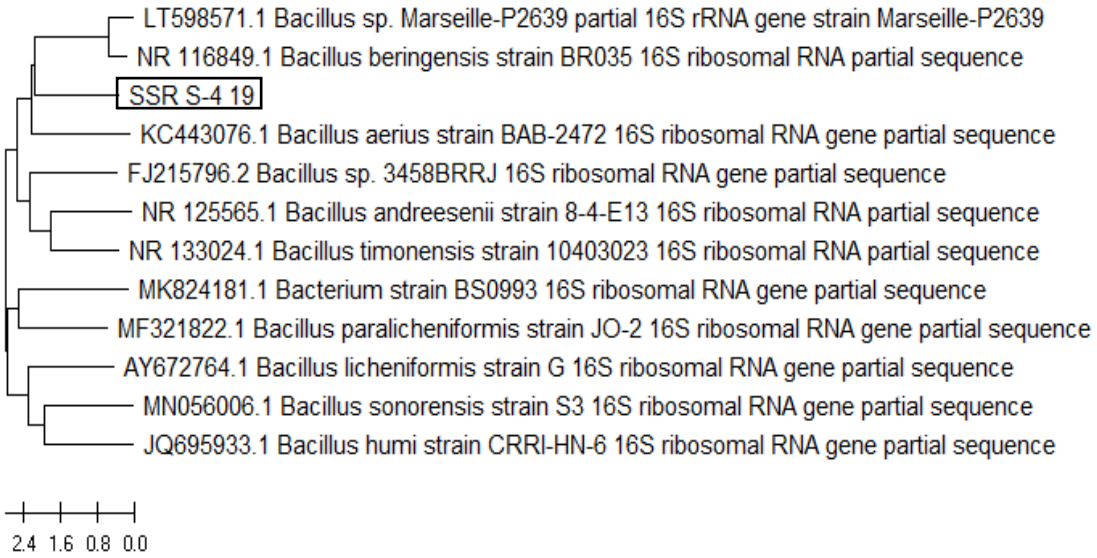


Figure 16: Evolutionary analysis by maximum evolution (SSR_S-4_19)

The evolutionary history was inferred using the Minimum Evolution method. The optimal tree with the sum of branch length = 24.16192832 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm at a search level of 1. The Neighbor-joining algorithm was used to generate the initial tree. This analysis involved 12 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1519 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

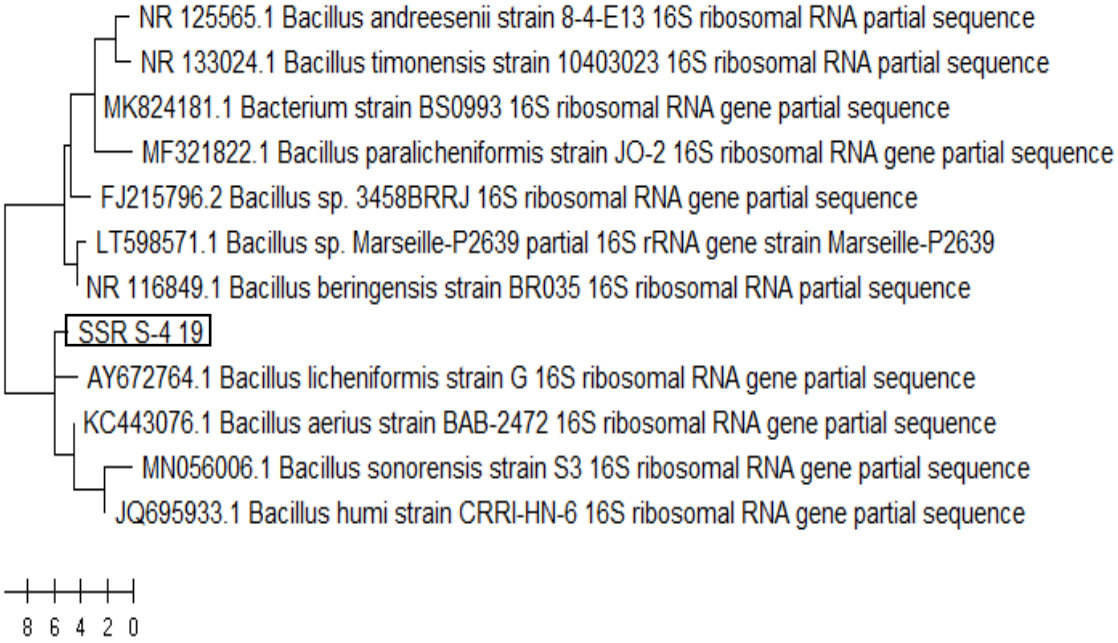


Figure 17: Evolutionary analysis by maximum likelihood method (SSR_S-4_19)

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood (-23815.65) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 12 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1519 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

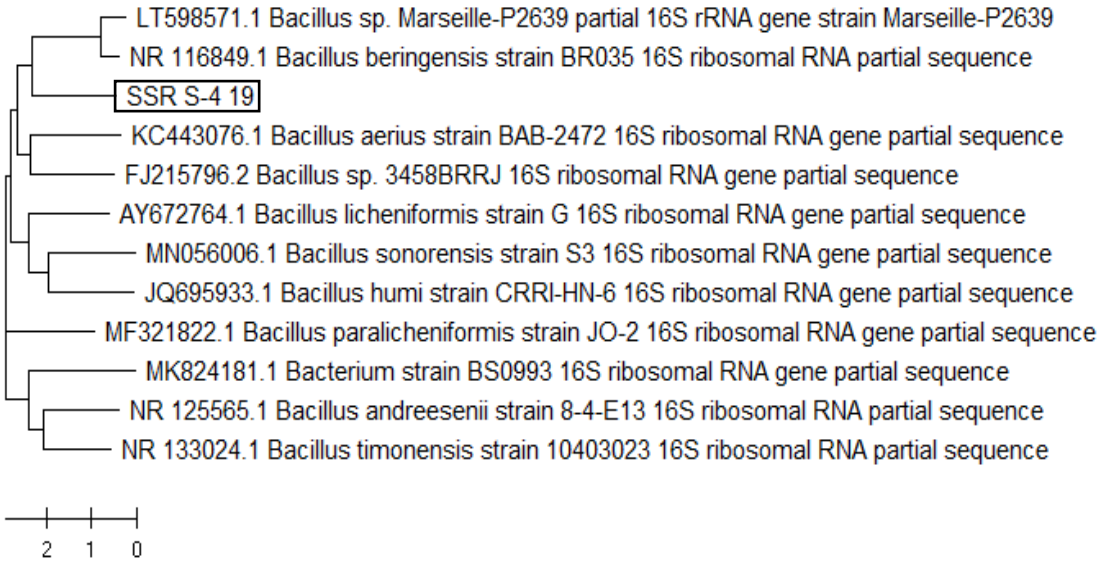


Figure 18: Evolutionary analysis by Neighbour-joining (SSR_S-4_19)

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 24.23292750 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 12 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1519 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

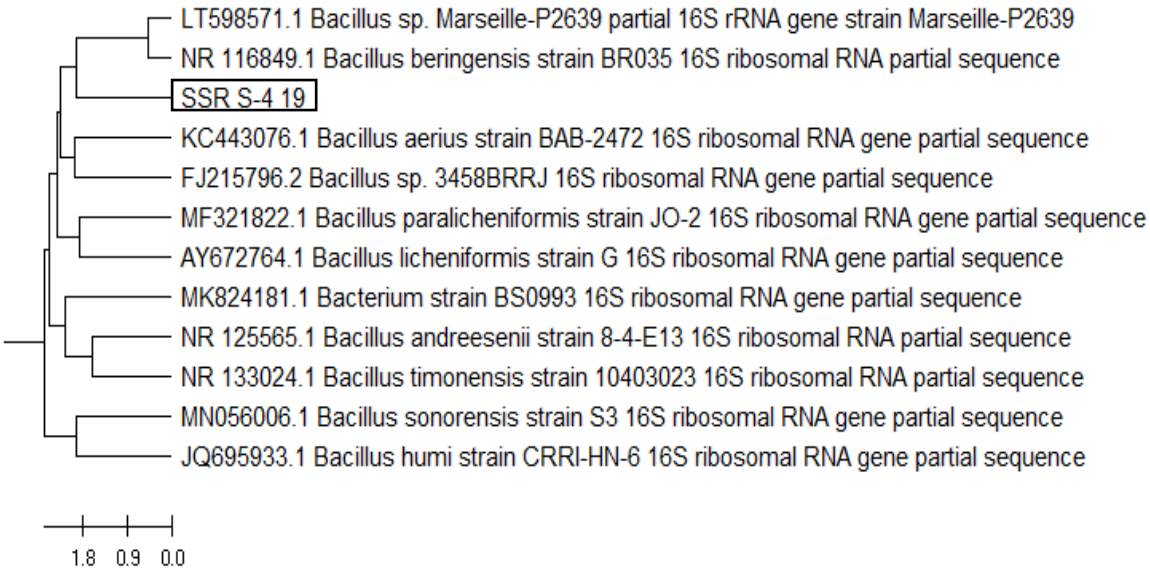


Figure 19: Evolutionary analysis by UPGMA (SSR_S-4_19)

The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 24.33800035 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 12 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1519 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

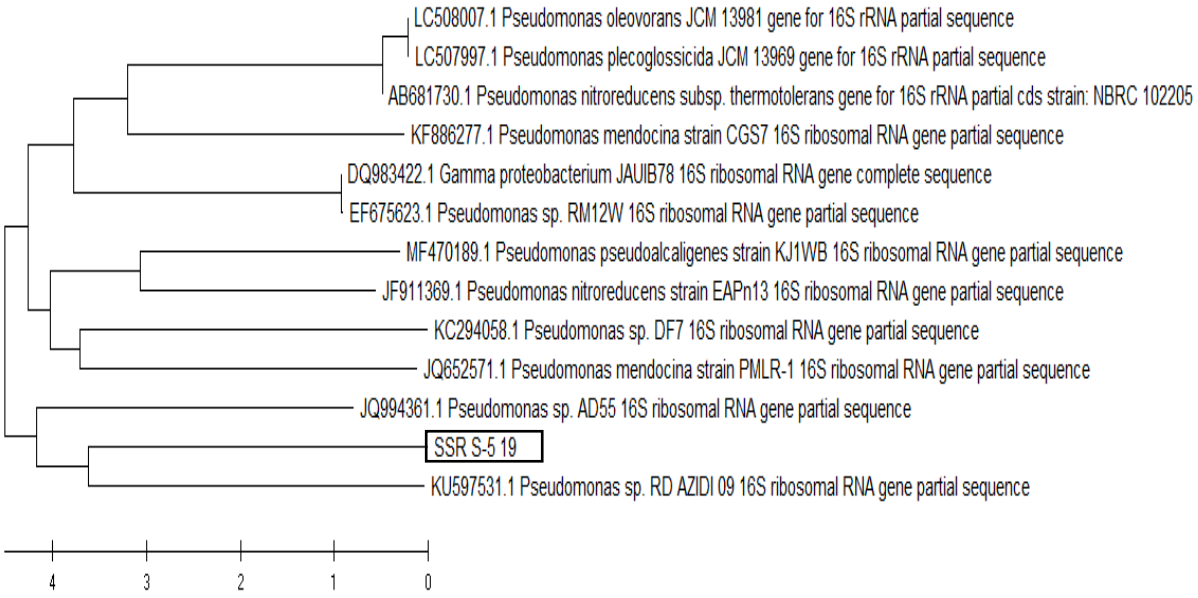


Figure 20: Evolutionary analysis by maximum evolution (SSR_S-5_19)

The evolutionary history was inferred using the Minimum Evolution method. The optimal tree with the sum of branch length = 15.79531876 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm at a search level of 1. The Neighbor-joining algorithm was used to generate the initial tree. This analysis involved 13 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1498 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

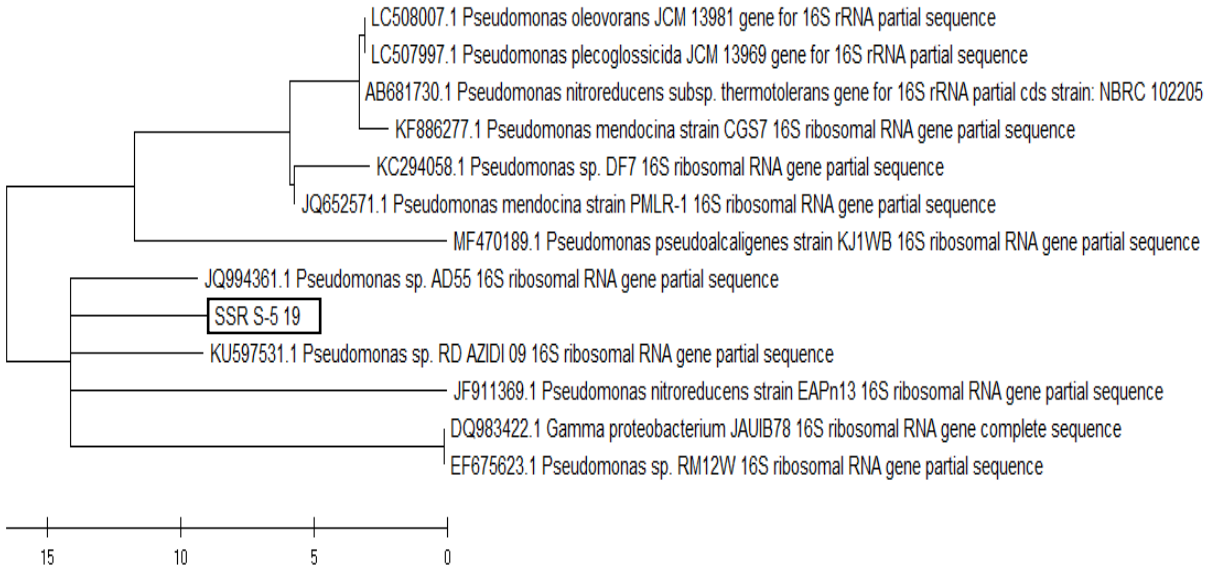


Figure 21: Evolutionary analysis by maximum likelihood method (SSR_S-5_19)

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood (-21326.24) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 13 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1498 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

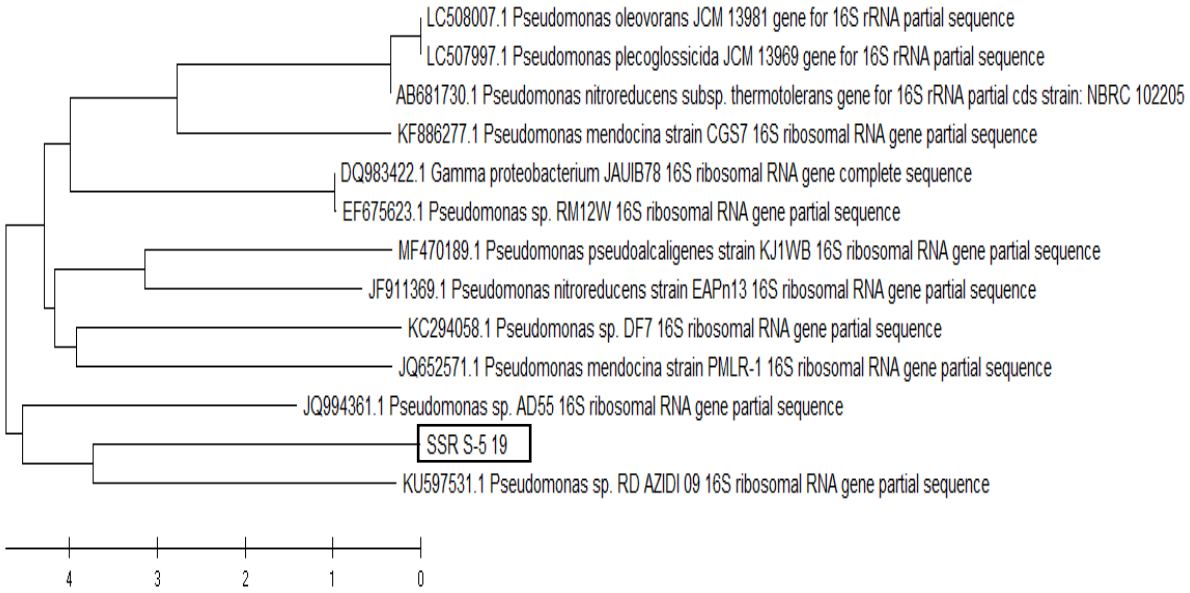


Figure 22: Evolutionary analysis by Neighbour-joining (SSR_S-5_19)

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 15.69878689 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 13 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1498 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

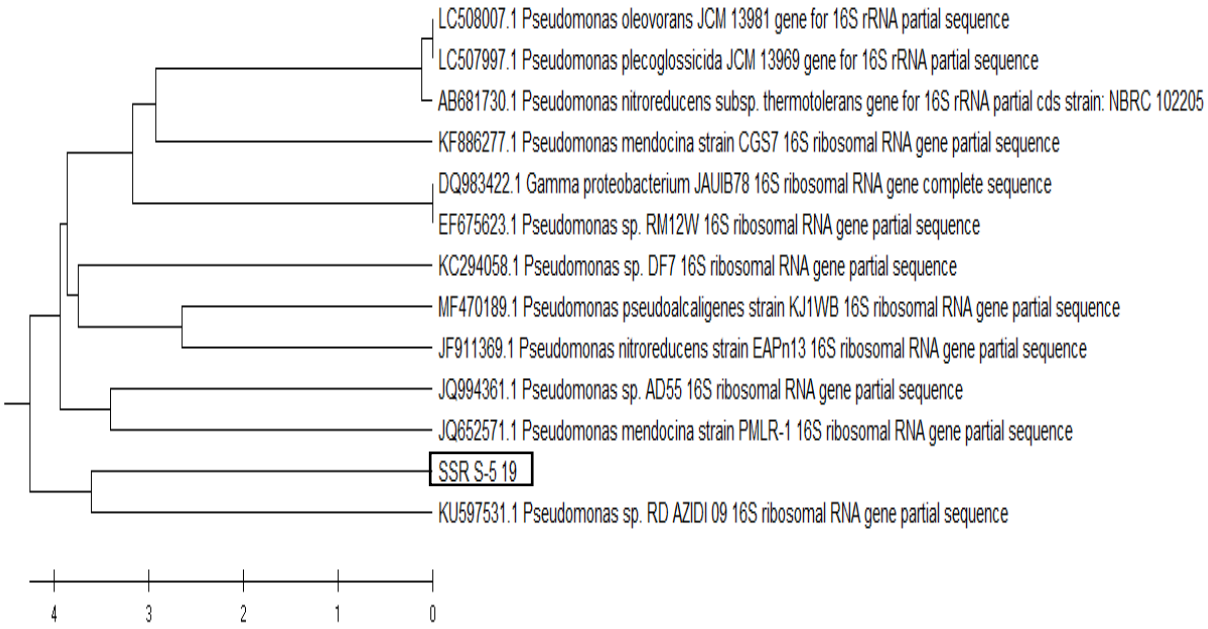


Figure 23: Evolutionary analysis by UPGMA (SSR_S-5_19)

The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 15.79676201 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 13 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1498 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

Chapter 4

Conclusion

On the whole, isolates found in this study showed efficiency in decolorizing the textile dye. According to phylogenetic tree analysis, isolates SSR_B-3_19, SSR_S-4_19 and SSR_S-5_19 was suspected to be *Bacillus salidurans*, *Bacillus* sp. Marseille and *Pseudomonas* sp. respectively. Among the isolates, SSR_S-4_19 was effective in decolorizing both dyes used in this project. In comparison with the decolorization rate, SSR_S-4_19 was more efficient in decolorizing Setazol Blue BB than SSR_S-5_19 whereas SSR_B-3_19 was more competent in decolorizing Black B than SSR_S-4_19. Considering the decolorization rates, all the three isolates were efficient in decolorizing the dyes. Since, the isolates were efficient decolorizer, it could be promising for future use in the decolorization or treatment of textile wastewater containing various dye.

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