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BIOREMEDIATION OF TEXTILE DYE EFFLUENT BY NEWLY ISOLATED *OCHROBACTRUM SPP.* AND THEIR BIOCHEMICAL AND MOLECULAR CHARACTERIZATION

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ABSTRACT

Textile factories discharge untreated harmful effluents into the environment which cause serious damage to the soil and water. The several bacteria capable of degrading textile dye reside naturally in the environment. The six different bacterial isolates were screened for dye decolouration capability. The decolouration studies were performed in liquid Nutrient Agar medium (NA) amended with different textile dyes, such as Spectron yellow F3RN, Spectron Navy CLBC, and SpectronRoseF3BN. The minimum inhibitory concentrations (MIC) were also examinedin this study at different concentrations. Out of six bacterial isolates, one bacterial isolate was performed well and taken for further studies. It was identified as *Ochrobactrum spp.* based on 16S rRNA sequence analysis as well as biochemical and morphology analysis. The optimal condition for the decolouration of three different dyes by *Ochrobactrum spp.* was at pH 7.0 and 35°C in 60 h of incubation. The bacterial sequence *Ochrobactrum spp.* was submitted to NCBI (National Centre for Biotechnological Information). The isolated bacterial strain *Ochrobactrum spp.* would be a potential candidate for microbes-based treatment to degrade dye from textile effluents.

***Keywords*:** 16S rRNA, BLAST, Phylogenetic tree, Degradation, Textile dye.

Introduction

The first man-made synthetic dye called mauevin was discovered in 1856 which contributes to the elimination of natural dye. Since then, over 100,000 dyes have been generated worldwide with an annual production of over 7×105 metric tonnes (Forster and Christie, 2013).Synthetic dyes are mostly used in textile dyeing or printing, paper printing, cosmetics, pharmaceuticals, leather industries, etc. A large amount of wastewater with contaminants have released directly into the environment from these industries (Babu *et al*., 2007). Untreated effluents are hazardous and very harmful to the flora and fauna life. These contaminated effluents are also carrying organic and inorganic materials such as a high quantity of Cl, Pb, Fe, Fl, and other heavy metals (Hill *et al*., 1993). Dyes are usually aromatic and heterocyclic compounds and are often recalcitrant, some of them being toxic and even carcinogenic (Vyas and Molitoris, 1995).

The contaminated effluents are treated by three main methods i.e., physical, chemical, and biological. Physico-chemical methods contain adsorption, coagulation, precipitation, filtration, oxidation, irradiation, incineration, and ultra-filtration. The main disadvantage of Physico-chemical methods was very difficult in handling and cost-effective (Patil *et al*., 2008). The biological method is the best alternative method for textile dye treatment (Hayase *et al*., 2000). In the biological method, microorganisms such as bacteria and fungi are involved and degrade or decolorize the dye which has been reported by many researchers. Recently reported bacterial strains showed high efficacy in removing textile dyes. They are *Pseudomonas sp*. SUK1 (Kalyani *et al*., 2009), *Pseudomonas aeurogenosa* NBAR12 for reactive Blue 172 (Kumar *et al*., 2016), *Rhizobium radiobatcter* MTCC8161 for reactive red 141 (Telke *et al*., 2008). The three textile azo dyes such as Reactive Red 198, Reactive Red 141, and Reactive Blue 214 were degraded by mixed fungal cultures reported in 2011(Carlos Nascimento *et al*., 2011). The dye Basic fuchsin (81.85%) followed by Nigrosin (77.47%), Malachite green (72.77%), and dye mixture (33.08%) were the textile dyes decolorized by *Aspergillus niger* reported in 2014 (Babita Rani *et al*., 2014).Degradation studieswere carried out in methyl orange and Eriochrome Balck T using basidiomycetes fungi and degraded more than 50% in 3 days and 33% respectively (Anuradha Kumari, 2015).In this study, we have isolated, screened, and characterizedthe dye-degrading bacteria collected from the Tripur textile industry, in Tamil Nadu.

Materials and Methods

Sample collection

The untreated textile effluents along with sewage were released into the soil in the Tripur textile industry, Tamil Nadu. The Soil samples were collected aseptically and preserved in sterile plastic bottles. It was stored at 4^0C in the refrigerator.

Isolation of dye-degrading bacteria from the dye-contaminated soil samples

Serial dilutions from 10^{-1} to 10^{-7} were prepared by pipette out the appropriate amount of distilled water suspension in 1 ml of dye-effluent soil sample. The nutrient agar plates were prepared and labelled. Then 0.1 ml of an aliquot from 10^{-6} and 10^{-7} dilutions was pipette out into the corresponding nutrient agar plate. Nutrient agar medium (Hi Media Laboratories Pvt. Ltd., Mumbai, India) was used for the isolation of degrading bacteria from the dye effluent. The sample was spread on the agar plate using the L-Rod (spread plate technique) and incubated at 37°C for 24 h. After incubation, bacterial colonies were observed and recorded.

Minimum Inhibitory Concentration (MIC) analysis

Randomlysix different colonies were selectedfor thisanalysis. The microorganisms were tested for their ability to produce visible growth in dye-containing Nutrient Agar Medium (NA medium) at different concentrations. Three dyes namely Spectron yellow F3RN, Spectron Navy CLBC, and Spectronrose F3BN were used for this study.

Growing isolated bacterial strains on NA medium containing dyes allowed researchers to calculate the minimum inhibitory concentration of each dye against those isolates. The concentration of the relevant dyes was steadily increased until the bacterial strain was unable to form a colony. The dyes had an initial concentration of 1%/100 ml. The culture growing at the initial concentration was then streaked onto the higher concentration of dye (5%/100 ml). The concentration at which bacterial strain failed to grow colony was considered as MIC.

Decolouration Assay

The dye-decolourizing bacterial isolates were inoculated into the boiling tube containing the liquid NA media (Hi Media Laboratories Pvt. Ltd., Mumbai, India) supplemented with three different dyes and incubated in an orbital shaker at 200 rpm/min for 7 days at 30^0C. The decolouration was observed every 24 h. The negative control consisted of an un-inoculated tube with a liquid NA medium. After that, the samples were analyzed based on spectrophotometer readings.

Morphological analysis

The selected six colonies were purified by repeated subculture on NA medium (Streak plate method). The selected colonies were subjected to gram staining and colony morphological analyses.

Biochemical analysis

The selected bacterial isolate was taken for biochemical analysis. Citrate, adonitol, and other 64 amino acid including sugar and enzyme tests also were carried out by BIOMÉRIEUX VITEK® 2 SYSTEM (Bio Line., Coimbatore) for the bacterial isolate identification.

Molecular characterization

Genomic DNA isolation:

The overnight culture of the selected bacterial isolate was taken for genomic DNA isolation by the CTAB method (Doyle and Doyle, 1990). The quality of genomic DNA was confirmed by Gel electrophoresis using 1.0% agarose gel and visualized under a UV spectrophotometer.

16S ribosomal RNA isolation and sequencing for bacterial identification

The 16S rRNA gene was amplified by PCR using universal primers (Forward primer 5'AGAGTTTGATCCTGGCTCAG3' and Reverse primer 5'AAGGAGGTGATCCAGCC 3'). The total 20µl of the cocktail mixture contained 2µl of template DNA, 10 µl of Dream Taq Green PCR Master Mix (Thermo Scientific), 0.5µl of forwarding primer, 0.5 µl of reverse primer, and 8µl of nuclease-free water. The PCR condition for 16S rRNA was 95˚C for 7 min (Initial denaturation) followed by 35 cycles of 95˚C for 1 min (Denaturation), 60˚C for 45 sec (Annealing), 72˚C for 45 sec, and a final extension at 72˚C for 15 min. PCR reaction was performed in Applied Biosciences (Veriti) thermocycler. Amplified PCR product was purified by gel elution kit (Gene JET Gel Extraction Kit-Thermo Scientific). The eluted16S rRNA fragment was subjected to partial sequence and the process was carried out in Chromous Biotech Pvt. Ltd, Bangalore.

BLASTn Analysis and Phylogenetic Analysis

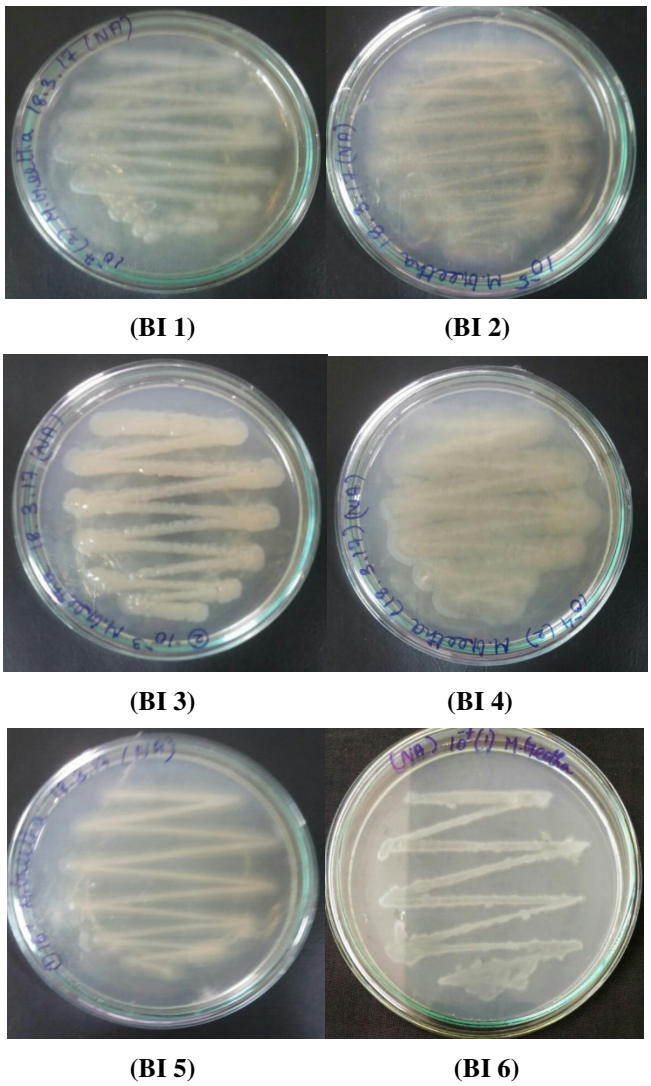
The sequences were analyzed using the BLAST (Basic Local Alignment Search Tool) tool (http://www.ncbi.nlm.nih.gov) for searching about homologysearch with other sequences deposited in Gene Bank. Highly similar sequences were selected from the NCBI Gene Bank and aligned using Clustal W multiple sequence alignment software (www.ebi.ac.uk/Tools/clustalw2). Bacterial phylogenetic trees were also constructed in BioEdit Sequence Alignment Editor and MEGA 4.0 software (Shah *et al*., 2016). The analysed sequence was deposited into NCBI.

Results and Discussion

Isolation of dye-degrading bacteria

The soil sample was collected from the dye-contaminated area and using the serial dilution method, the six different dye-degrading bacteria were isolated and maintained in the NA media shown in (Figure 1). Similarly, it has been reported that five different bacterial species, *Bacillus subtilis, Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pneumoniae,* and *Escherichia coli* were isolated from the textile dye effluent (Saranraj *et al*., 2010). It

has also been reported that three different bacterial species, *Bacillus sp., E. coli,* and *P. fluorescens* were isolated from textile dye effluent-contaminated soil (Sriram *et al*., 2013).



(BI 1) **(BI 2)**

(BI 3) **(BI 4)**

(BI 5) **(BI 6)**

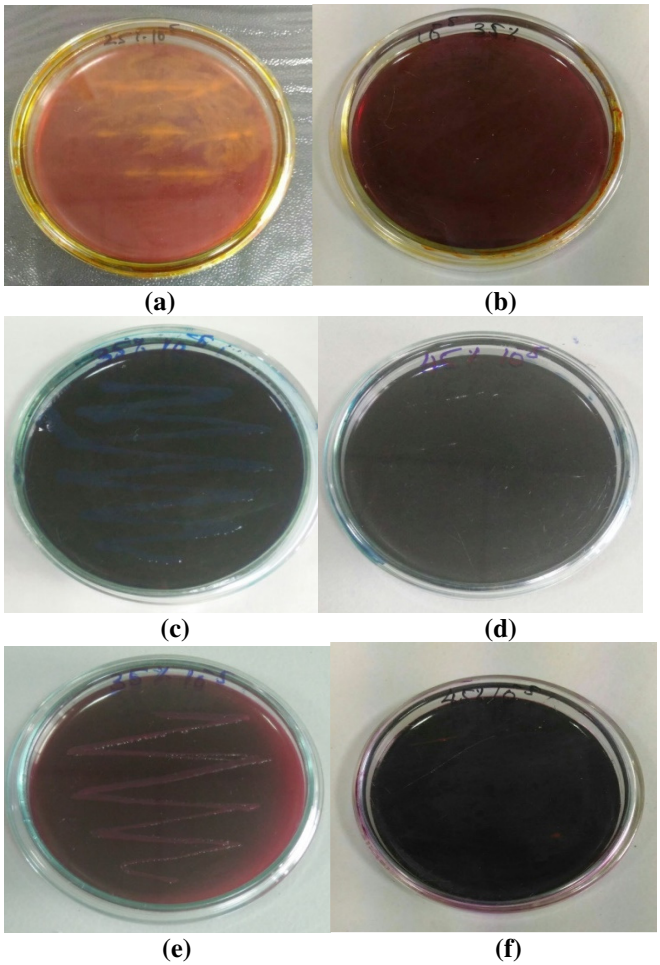
Fig. 1 : Six Different bacterial isolates collected from dye-contaminated soil.

Minimum Inhibitory Concentration (MIC) analysis

To identify the susceptible capacity of dye-decolourisingbacteria, the bacterial isolates were sub-cultured into the dye-containing NA medium at different concentration levels i.e., 1, 1.5, 2.0, 2.5, 3.0, 3.5, 4.5 and 5.0 % were shown in (Table 1 and Figure 2). MIC study of each dye showed that all six bacterial isolates were tolerating the dye concentration between 1 to 3.5% (Table 1) and all six bacterial strains have the highest tolerance to all three dyes. MIC study revealed that all six selected bacteria have a high tendency to tolerate and grow under a dye-stressed environment.

Decolouration Assay:

Based on spectrophotometer readings, it was found that the isolates BI 4 showed maximum hydrolysis whereas BI 1, BI 2, BI 3,BI 5, and BI 6 isolates showed slight hydrolysis in a liquid broth (Table 2). There was no decolouration observed in the control. Thus they were not selected for further study. The desired bacterial isolate (BI 4) with strong decolourising ability was selected for further analysis. The optimal condition for the decolouration of three different dyes by *Ochrobactrum spp.* was at pH 7.0 and 35°C in 60 h of incubation. The metabolic processes and interactions between these strains of bacteria might be the reason for the effluent's effective and rapid decolorization (Phugare *et al*., 2011; Murugalatha *et al*., 2010; Togo *et al*., 2008). The bacteria, *Lysinibacillus spp*. required 96 h (Saratale *et al*., 2013) and *Phanerochaetes ordia* required 48 h (Harazono and Nakamura, 2005) for optimum decolourization of dyes.



(a) **(b)**

(c) **(d)**

(e) **(f)**

Fig. 2 : Minimum Inhibitory Concentration analysis (MIC). Culture plates a, b, c, d, e, and f showing growth of bacterial strain (BI 4) on Nutrient Agar medium incorporated with different concentrations of dyes.

Morphological characterization

Gram staining tests and colony morphological analysiswere done for the six different isolates and results showed that the selected bacterial isolate (BI4) was gram-positive bacteria shown in (Table 3). The BI 4 was white, rounded end and circular shaped bacteria.

Biochemical characterization

Bacterial isolate(BI4) showed negative results of D-Cellobiose, D-Glucose, D-Maltose, and D-Manose and positive results of Adonitol, urease, citrate (sodium), and succinate alkalinization as shown in (Table 4). Therefore with the above results, the bacterial isolate BI 4 was confirmed as *Ochrobactrum spp*..

Molecular characterization

The genomic DNA was isolated from the selected bacterial isolate (BI4) with three replicates and 16 S rDNA was amplified from three replicates using PCR.The amplified

PCR product was ~1500bp shown in (Figure 3). A similar result was reported by Iwana and Azrimi, (2013). The sequences were analyzed using the BLAST tool. The output of BLAST results showed that >99.50% similarity was obtained with *Bacillus anthracis, Bacillus thuringiensis,* and *Bacillus cereus.* A similar result was reported by Kumar *et al*., (2016). The sequence of *Ochrobactrum spp.* was submitted to Gene bank and the accession number was MW577354. Finally, a phylogenetic tree was constructed with other similar sequences from the NCBI database shown in (Figure 4).

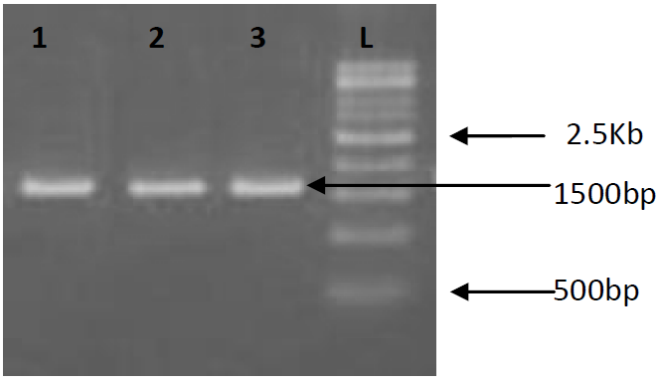


Fig. 3 : PCR products loaded on 1% Agarose Gel

Lane Description:

1. Sample-1 (BI 4; Replicate 1)
2. Sample-2 (BI 4; Replicate 2)
3. Sample-3 (BI 4; Replicate 3)

L- 500 bp ladder

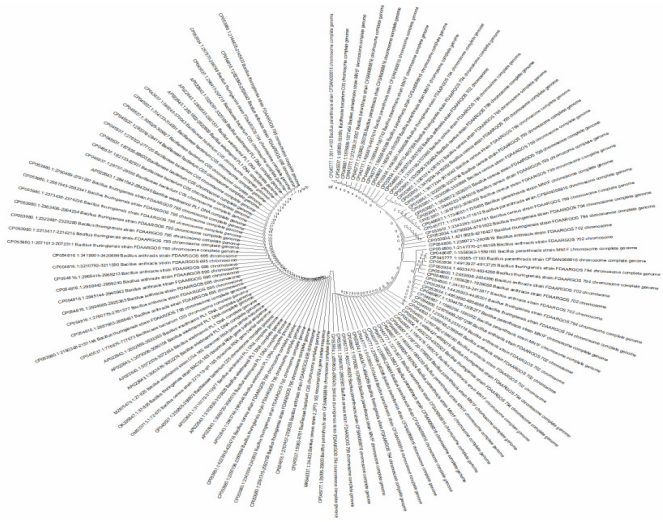


Fig. 4: Phylogenetic tree of 16S rRNA gene sequences of dye degrading isolate and close relative reference isolates retrieved from the database with an accession number.

Conclusion

In this study, the newly isolated bacteria *Ochrobactrum spp*.. has demonstrated potential for its dye decolourtion. The optimal condition for the decolouration of three different dyes by *Ochrobactrum spp.* was at pH 7.0 and 35°C in 60 h of incubation. It can be concluded from the overall findings that the isolated bacteria *Ochrobactrum spp.* could effectively be used as an alternative to the physical and chemical processes of textile effluents as they have a high potential for being able to decolorize or degrade dyes.

Table 1: Minimum Inhibitory Concentration (MIC) analysis using six different bacterial isolates:

Dye Used/ Different concentrations of dyes		Bacterial Isolates (BI)					
		BI 1	BI 2	BI 3	BI 4	BI 5	BI 6
Spectron Yellow F3RN	1.0 %	+	+	+	+	+	+
	1.5%	+	+	+	+	+	+
	2.0 %	+	+	+	+	+	+
	2.5 %	+	+	+	+	+	+
	3.0 %	+	+	+	+	+	+
	3.5 %	-	-	-	-	-	-
	4.0 %	-	-	-	-	-	-
	4.5 %	-	-	-	-	-	-
	5.0 %	-	-	-	-	-	-
Spectron Navy CLBC	1.0 %	+	+	+	+	+	+
	1.5%	+	+	+	+	+	+
	2.0 %	+	+	+	+	+	+
	2.5 %	+	+	+	+	+	+
	3.0 %	+	+	+	+	+	+
	3.5 %	+	+	+	+	+	+
	4.0 %	-	-	-	-	-	-
	4.5 %	-	-	-	-	-	-
	5.0 %	-	-	-	-	-	-
Spectron Rose F3BN	1.0%	+	+	+	+	+	+
	1.5%	+	+	+	+	+	+
	2.0 %	+	+	+	+	+	+
	2.5 %	+	+	+	+	+	+
	3.0 %	+	+	+	+	+	+
	3.5 %	+	+	+	+	+	+
	4.0 %	-	-	-	-	-	-
	4.5 %	-	-	-	-	-	-
	5.0 %	-	-	-	-	-	-

'+' – Presence of bacterial growth

'-' – No bacterial growth

Table 2: Dye decolourising ability analysis based on visual identification by using different bacterial isolates (BI 1, BI 2, BI 3, BI 4 BI 5, and BI 6).

Bacterial isolate (BI)	Spectron yellow F3RN	Spectron Navy CLBC	Spectron rose F3BN	Control
BI 1	+	++	+	----
BI 2	+	+	+	----
BI 3	----	+	---	----
BI 4	+++	+++	+++	----
BI 5	+	+	----	----
BI 6	----	----	+	----

*'**----**' - No decolourisation; '**+**' - Slight decolourisation;'**++**'- Moderate decolourisation and '**+++**'-Intense decolourisation

Table 3: Morphological characterization of six different bacterial isolates:

Colony morphology / Bacterial isolate (BI)	Shape	Configuration	Elevation	Colour	Spore	Gram stain
BI 1	Rods in chain	Circular Lobate	Flat	White	No Spore	Positive
BI 2	Rods in chain	Circular	Convex	Off- White	No Spore	Positive
BI 3	Rods	Circular	Slightly raised	Mucoid	No Spore	Positive
BI 4	Straight rods	Circular	Rounded ends	White	No spore	Positive
BI 5	Cocci shape in tetrad	Circular	Convex	Bright Yellow	No spore	Positive
BI 6	Rods	Circular	Raised	White	No spore	Positive

Table 4: Biochemical analysis performed on bacterial isolate (BI 4).

S.No	Well	Mnemonic	Name of the Test	Result
1	2	APPA	Ala-Phe-Pro-ARYLAMIDASE	-
2	3	ADO	ADONITOL	+
3	4	Pyra	L-Pyrrolydonyl-ARYLAMIDASE	+
4	5	IARL	L-ARABITOL	-
5	7	dCEL	D-CELLOBIOSE	-
6	9	BGAL	BETA-GALACTOSIDASE	-
7	10	H2S	H2S PRODUCTION	-
8	11	BNAG	BETA-N-ACETYL-GLUCOSAMINIDASE	-
9	12	AGLTp	GlutamylArylamidasePna	+
10	13	dGLU	D-GLUCOSE	-
11	14	GGT	GAMMA –GLUTAMYL-TRASEFERASE	-
12	15	OFF	FERMENTATION/GLUCOSE	-
13	17	BGLU	BETA GLUCOSIDASE	-
14	18	dMAL	D-MALTOSE	-
15	19	dMAN	D-MANNITOL	-
16	20	dMNE	D-MANNOSE	-
17	21	BXYL	BETA-XYLOSIDASE	-
18	22	BAlap	BETA-AlaninarylamidasePna	-
19	23	ProA	L-Proline ARYLAMIDASE	+
20	26	LIP	LIPASE	-
21	27	PLE	PALATINOSE	-
22	29	TyrA	Tyrosine ARYLAMIDASE	+
23	31	URE	UREASE	+
24	32	dSOR	D-SORBITOL	-
25	33	SAC	SACCHAROSE/SUCROSE	-
26	34	Dtag	D-TAGATOSE	+
27	35	Dtre	D-TREHALOSE	-
28	36	CIT	CITRATE(SODIUM)	+
29	37	MNT	MALONATE	-
30	39	5KG	5-KETO-D-GLUCONATE	-
31	40	ILATk	L-LACTATE alkalinisation	+
32	41	AGLU	ALPHA-GLUCOSIDASE	-
33	42	SUCT	SUCCINATE alkalinisation	+
34	43	NAGA	Beta-N-ACETYL-GALACTOSAMINIDASE	-
35	44	AGAL	ALPHA-GALACTOSIDASE	-
36	45	PHOS	PHOSPHATASE	-
37	46	GlyA	Glycine ARYLAMIDASE	+

38	47	ODC	ORNITHINE DECARBOXYLASE	-
39	48	LDC	LYSINE DECARBOXYLASE	-
40	52	ODEC	DECARBOXYLASE BASE	
41	53	IHISa	L-HISTIDINE assimilation	-
42	56	CMT	COUMARATE	-
43	57	BGUR	BETA-GLUCORONIDASE	-
44	58	O129R	O/129 RESISTENCE (Comp.vibrio.)	-
45	59	GGAA	Glu-Gly-Arg-ARYLAMIDASE	-
46	61	IMLTa	L-MALATE assimilation	+
47	62	ELLM	ELLMAN	+
48	64	ILATa	L-LACTATE aaimilation	-

* '+' – Positive and '-' – Negative

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