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Decolorization of Textile Dye Effluent by Genetically Improved Bacterial Strains

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Abstract

Synthetic dyes are released into the environment from textile industrial effluents. The discharge of this colored wastewater into rivers and lakes leads to a reduction in sunlight penetration in natural water bodies, which, in turn, decreases both photosynthetic activity and dissolved oxygen concentration and is toxic to living beings. Bacterial isolates are optimized for growth and biomass production before using them for decolorizing dye effluent. The bacterial isolates *Bacillus* sp. 1 and *Bacillus* sp. 2 were employed at different percentages by volume with standard nutrient concentration. Of these bacterial isolates *Bacillus* sp. 2 recorded maximum color reduction. The pH and electrical conductivity (EC) were reduced in the decolorized effluent, and a reduction in biologic oxygen demand, chemical oxygen demand, total suspended solids, and total dissolved solids (TDS) were also observed.

Index Entries: Azodye; biodegradation; decolorization; effluent; mutants.

Introduction

In the United States, the textile industry is one of the country's largest industries, earning a large amount of foreign exchange and attracting public attention from the viewpoint of pollution. Untreated effluent from dyestuff production and dyeing mills is highly colored and hence objectionable if discharged into open waters. Even though the dye concentration may be well below 1 ppm, i.e., lower than many other chemicals found in wastewater, the color is predominant and visible. Approximately 10,000 different dyes and pigments *(1)* are used industrially.

Few industries have joined hands to chemically treat the effluent by common effluent treatment plants, and even after the treatment, the effluent possesses enormous soluble salts, which makes it unsuitable for

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agriculture *(2)*. Biologic treatment methods are cheap and often the best alternative with proper analysis and environmental control. However, genetic improvement of bacterial strains *(3)* for efficient and faster degradation needs to be studied. In the present study, textile dye factory effluent was characterized. The effect of standard culture and isolated cultures on dye effluent decolorization was studied. The conditions for the growth of isolated microorganism *(4)* in decolorization of textile dye effluent were optimized. The isolated strains were genetically improved for enhanced decolorization of textile dye *(5)* effluent through mutagenesis.

Materials and Methods

Isolation of Bacteria

To ascertain a certain microbial culture for dye effluent decolorization, soil samples were collected from the tannery effluent drenched areas located at Chennai, India. The acclimatized microorganisms were isolated by employing a standard serial dilution plating technique *(6)*.

Ten-gram soil samples and 10-mL water samples were taken in 90-mL sterile water blanks and serially diluted to 10^6 times using a sterile water blank. After thorough shaking, a 1-mL aliquot from 10^6 dilutions was drawn and pour plated in nutrient agar medium (Waksman and Fred, 1922). The plates were incubated at 35°C in an incubator for 24 h for bacteria *(15)*. The isolated bacteria were further purified by subsequent subculturing *(7)* and maintained in the slant culture at 4°C.

Screening of Elite Microbes for Decolorization

Dye factory effluent was scanned in an ultraviolet (UV) spectrophotometer (Elico SL-159 UV spectrophotometer) to ascertain the wavelength, and maximum absorbance was observed at 510 nm and the rate of decolorization was monitored at this wavelength. The bacterial cultures *Bacillus* sp. 1 and *Bacillus* sp. 2 isolated from contaminated soil were used for the decolorization of dye effluent.

Cell Morphology

The isolates of bacteria were grown in a nutrient broth for 48 h in a 100-mL Erlenmeyer flask on a shaker (Orbitek). The cell morphology of the isolates was observed after simple staining with crystal violet, and the cells were observed under oil immersion objective of a research microscope (Tokyo, Japan).

Biochemical Characteristics of Bacterial Isolates

Gram's reaction was performed, and the presence or absence of endospores was observed by spore staining per the standard method. The following biochemical tests were carried out according to the method shown in Table 1 *(16)*.

Table 1
Characterization of Bacterial Isolates

Sl. no.	Characteristic	*Bacillus* sp. 1	*Bacillus* sp. 2
	A. Morphologic characteristics		
1.	Shape	Short rod	Short rod
2.	Cell size	0.8–1.2 micron	1.8–2.0 micron
3.	Gram staining	+	+
4.	Spore staining	+	+
	B. Biochemical test		
1.	Catalase activity	+	+
2.	Indole test	+	+
3.	Methyl red	+	+
4.	Gelatin hydrolysis	+	+
5.	Lactose utilization		
5a.	Gas production	–	–
5b.	Acid production	+	+

Gelatin Liquefaction

The ability of the bacterial isolates to produce gelatinase enzyme, which liquefies gelatin, was tested. The cultures were inoculated into nutrient gelatin deep tubes and incubated for 48 h at 28°C. Then the tubes were placed in a refrigerator at 4°C for 30 min and observed for gelatin liquefaction.

Mutagenesis (17)

To increase the decolorization efficiency, mutagenesis was carried out. The cell pellets were transferred to 100 mL of sterile distilled water and further diluted to obtain 10^9 colonies/mL. The population of colonies *(8)* was calculated by pour plate technique using NA medium. Four milliliters of the cell suspension was added to 95 mL of sterile distilled water in a 250-mL conical flask. A calculated quantity (200 ppm) of *N*-methyl *N*-nitro *N*-nitroso guanidine (NTG) was added to make up the volume to 100 mL. The flask was incubated over a water bath maintained at 37°C with occasional shaking for 60 min. After incubation 100 mL of prechilled (5 to 6°C) sterile distilled water was added to the flask to arrest the action of the mutagen. A 1-mL aliquot was pipetted out of the reaction mixture and diluted 10^6. One milliliter of the diluted sample was plated on nutrient agar (NA) medium. The plates were incubated for 48 h and observed for the growth of colonies.

In addition to cell dilution and plating, a 1-mL aliquot of the cell suspension was aseptically pipetted out into 100 mL of NA broth and kept over a shaker for 48 h to permit as much recombination as possible to take place. After incubation a 1-mL aliquot was diluted suitably in sterile water blanks

and plated in NA medium to determine the population. From the developing bacterial colonies the mutants were scored. The clones were evaluated for antibiotic resistance and decolorizing efficiency. The clones were plated on NA medium containing 250 ppm of streptomycin. From the flask, 0.5 mL of the cell suspension was aseptically transferred to the antibiotic-containing plates, spread uniformly using a glass spreader, and incubated for 24–48 h at 37°C. The number of colony-forming units on the two sets of antibiotic-containing plates was then counted. From this the frequency of mutation *(9)* was calculated for 250 ppm of antibiotics. The well-defined colonies on the 250-ppm plates were selected and streaked on NA medium. After discarding the revertants and leaky mutants, the stable colonies were streaked continuously on NA medium containing antibiotics for five generations to stabilize characters.

Characterization of Mutants (M)

Mutants were grown in NA medium for 24–48 h at 35°C. After good growth, the mutants and the wild strains were inoculated into 100 mL of NA broth in a 250-mL flask. The flasks were incubated for 16 h at 35°C under shaking condition. The flask was drawn at 4-h intervals for 16 h and optical density (OD) values were recorded for mutants and wild type at 610 nm.

Results and Discussion

Physicochemical and Biologic Characteristics of Dye Effluent

The physicochemical *(10)* and biologic characteristics *(11)* of dye effluent were analyzed over a period of 6 mo at 30-d intervals. The dye effluent showed variation in the physicochemical parameters during every sampling *(12)*. The color of the dye effluent was dark red. The pH and EC of the dye effluent were 8.37 and 10.8 d/Sm, respectively. The TDS and total suspended solids (TSS) of the dye effluent were 5810 and 4440 mg/L, respectively. The organic carbon content of the effluent was 5.45%. The total nitrogen and phosphorus in the dye effluent were present at a level of 441 and 15.47 mg/L, respectively.

Calcium, magnesium, sodium, potassium, chloride, and sulfate were present at a level of 521, 192.6, 719, 25.3, 686, and 671 mg/L, respectively. Carbonate and bicarbonate were present at a level of 12.5 and 3.23 mg/L, respectively. Micronutrients such as manganese and copper were recorded at a level of 0.121 and 0.066 mg/L, respectively. In addition, the microbial population of bacteria was present at a population level of 51×10^6 mL.

Characterization and Identification of Decolorizing Bacterial Cultures

Based on the biochemical tests *(13)*, the isolated bacterial cultures were identified as *Bacillus* sp. 1 and *Bacillus* sp. 2.

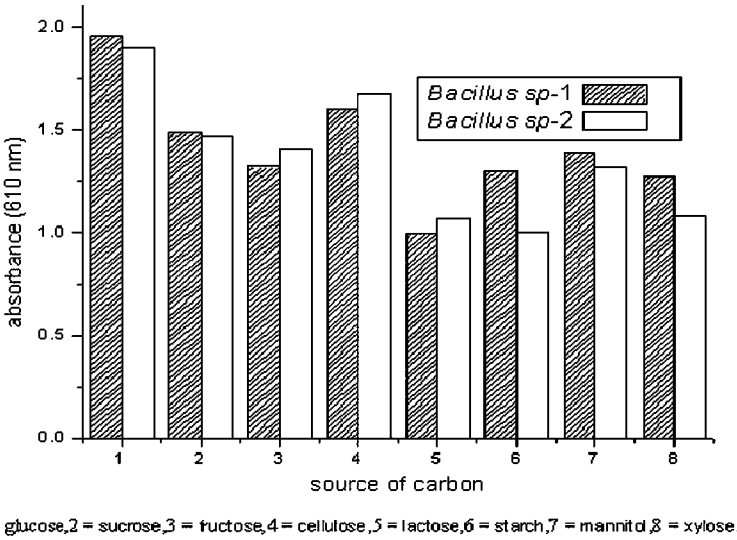


Fig. 1. Effect of different carbon sources on growth of *Bacillus* sp. 1 and 2: 1, glucose; 2, sucrose; 3, fructose; 4, cellulose; 5, lactose; 6, starch; 7, mannitol; 8, xylose.

Effect of Different Carbon Sources on Growth of Bacillus *sp. 1 and* Bacillus *sp. 2*

With respect to the *Bacillus* sp. 1, among eight carbon sources, glucose had a maximum OD of 1.956 compared with the other carbon sources. Cellulose and fructose had considerable growth, with ODs of 1.496 and 1.391, respectively, compared with glucose. Similarly, in *Bacillus* sp. 2, glucose had a maximum OD of 1.957. Cellulose and sucrose had considerable growth, with ODs of 1.489 and 1.401, respectively. Figure 1 presents the effect of the carbon source on the growth of *Bacillus* sp. 1 and 2.

Effect of Different Nitrogen Sources on Growth of Bacillus *sp. 1 and* Bacillus *sp. 2*

In general, organic sources of nitrogen supported better growth of bacteria than inorganic sources *(14)*. In *Bacillus* sp. 1, peptone and yeast extract had the highest ODs, 1.981 and 1.963, respectively. Similarly, in *Bacillus* sp. 2, peptone and yeast extract had the highest ODs, 1.971 and 1.903, respectively. Among the inorganic sources of nitrogen, a higher growth rate (OD 1.687 and 1.637, respectively) was recorded in *Bacillus* sp. 1 and 2 when ammonium chloride was used. Figure 2 presents the results.

Effect of Different pH on Growth of Bacillus *sp. 1 and* Bacillus *sp. 2*

Both the bacterial cultures *Bacillus* sp. 1 and 2 had maximum growth at pH 7.0, with ODs of 1.957 and 1.929, respectively. Similarly, at pH 8.0,

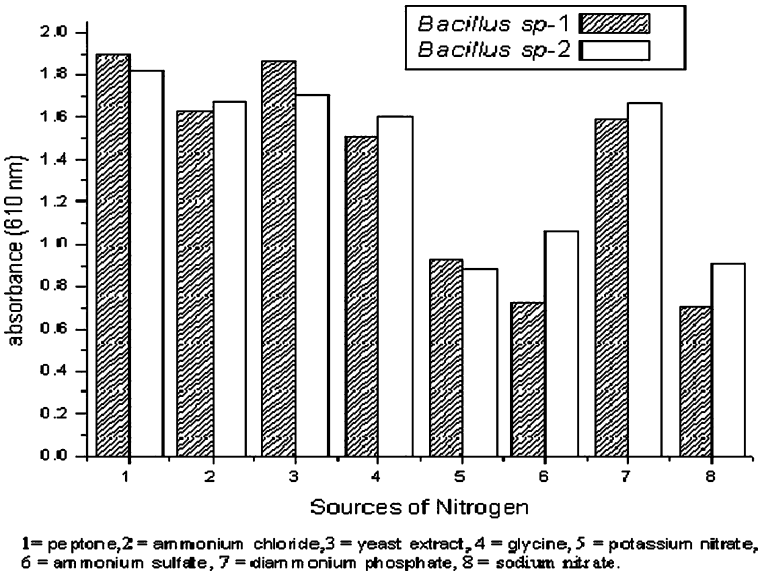


Fig. 2. Effect of different nitrogen sources on growth of *Bacillus* sp. 1 and 2: 1, peptone; 2, ammonium chloride; 3, yeast extract; 4, glycine; 5, potassium nitrate; 6, ammonium sulfate; 7, diammonium phosphate; 8, sodium nitrate.

Bacillus sp. 1 and 2 had ODs of 1.801 and 1.821, respectively. Figure 3 presents the effect of pH on the growth of the bacterial cultures.

Effect of Different Temperature on Growth of Bacillus *sp. 1 and* Bacillus *sp. 2*

The bacterial cultures *Bacillus* sp. 1 and 2 had maximum growth at 35°C, with ODs of 1.839 and 1.901, respectively. At 25°C, *Bacillus* sp. 1 and 2 had ODs of 1.691 and 1.707, respectively. At a high temperature of 45°C, *Bacillus* sp. 1 and 2 had the lowest growth, with ODs of 1.187 and 1.271, respectively. Figure 4 presents the effect of temperature on the growth of the bacterial cultures.

Genetic Improvement in Isolated Bacterial Cultures of Bacillus *1 and* Bacillus *2 for Color Reduction*

Mutagenesis in *Bacillus* sp. 1 and 2 yielded interesting mutants. Survival concentration (LD_{50}) of NTG to *Bacillus* sp. 1 and 2 was 200 $\mu g/mL$. The mutation frequency observed was 0.18 and 0.16% in *Bacillus* sp. 1 and 2, respectively.

Evaluation of Mutants for Fast Growth and Decolorization Efficiency

The growth of mutants of *Bacillus* sp. 1 and *Bacillus* sp. 2 was faster than that of the wild type. Mutants of bacterial cultures 1 and 2 were selected

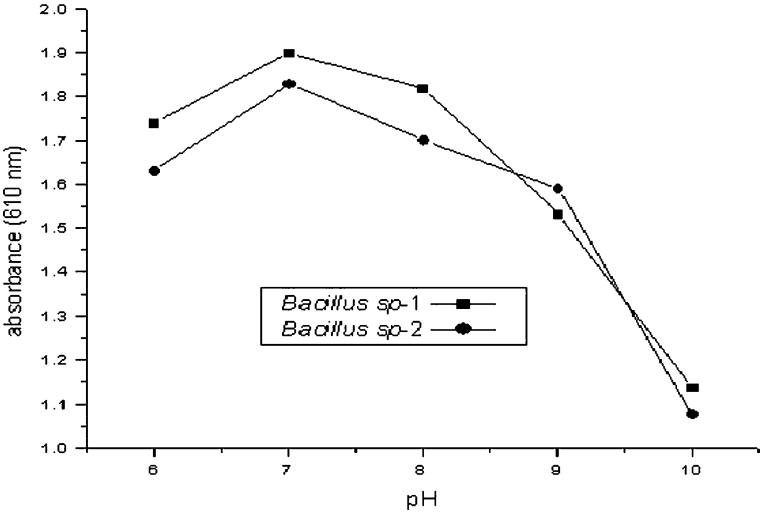


Fig. 3. Effect of different pH values on growth of *Bacillus* sp. 1 and 2.

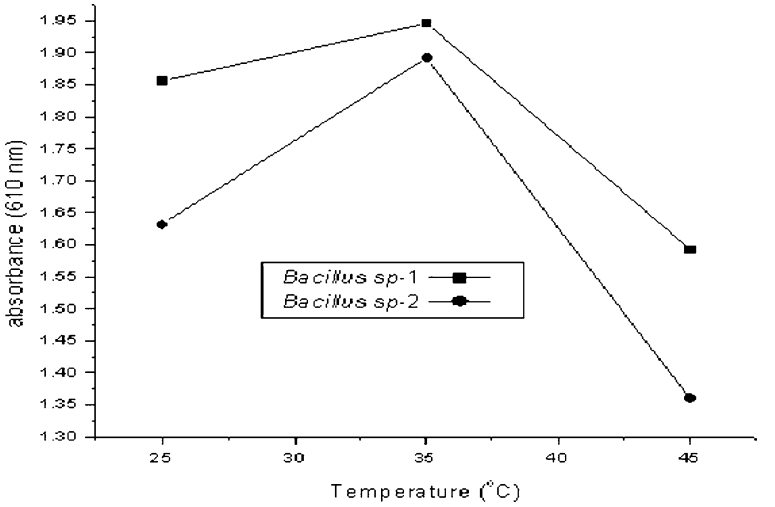


Fig. 4. Effect of different temperatures on growth of *Bacillus* sp. 1 and 2.

and grown for four generations to test antibiotic resistance *(13)* (streptomycin). The leaky mutants were discarded and stable mutants were selected. The mutants of *Bacillus* sp. and 2 (200 ppm of streptomycin) showed increased resistance to the antibiotic compared with their wild types (100 ppm of streptomycin). Mutants of *Bacillus* sp. 1 and 2 had maximum growth (OD 1.979 and 1.935, respectively) compared with wild types of *Bacillus* sp. 1 and 2 (OD 1.817 and 1.791, respectively) (Fig. 5).

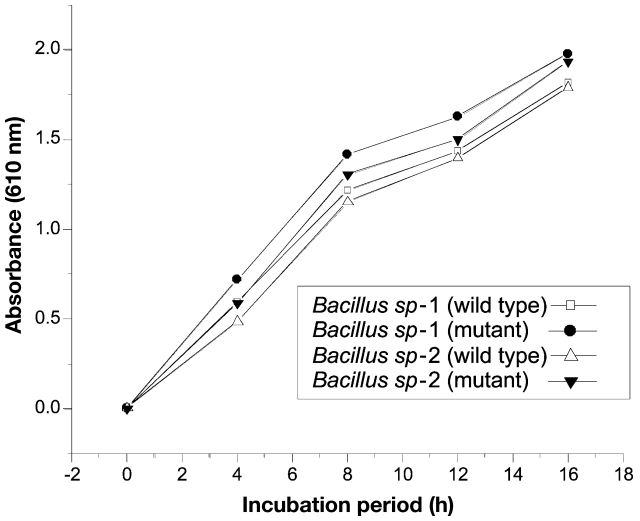
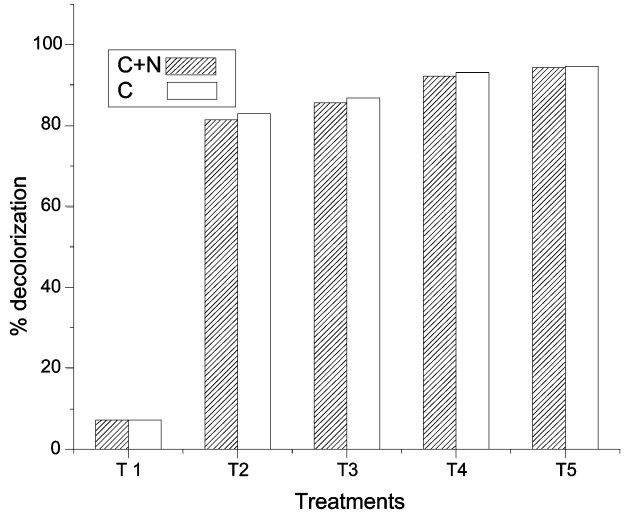


Fig. 5. Evaluation of mutants for fast growth of *Bacillus* sp. 1 and 2 and decolorization efficiency.



T1 = control, T2 = *Bacillus* sp-1, T3 = *Bacillus* sp-2, T4 = *Bacillus* sp-1M, T5 = *Bacillus* sp-2M. (M=Mutant)

Fig. 6. Decolorization of dye effluent by isolated bacterial cultures and their mutants with addition of carbon and carbon + nitrogen source: T1, control; T2, *Bacillus* sp. 1; T3, *Bacillus* sp. 2; T4, *Bacillus* sp. 1M; T5, *Bacillus* sp. 2M (M = mutant).

Decolorization of Dye Effluent by Isolated Bacterial Cultures and Their Mutants With Addition of Carbon and Carbon + Nitrogen Source

The color of the dye effluent was removed using bacterial isolates and their mutants. Color reduction in the dye was measured at 12-h intervals for 72 h. All the treatments irrespective of nitrogen addition showed signifi-

cant reduction of color in the dye effluent. The color reduction increased with an increase in incubation time. Among the treatments, *Bacillus* sp. 2M + carbon and nitrogen source and *Bacillus* sp. 2M + carbon source resulted in maximum color reduction of 94.65 and 93.05%, respectively, followed by *Bacillus* sp. 1M + carbon and nitrogen source and *Bacillus* sp. 1M + carbon source, which resulted in a reduction of 94.51 and 92.17%, respectively. Among the wild types, *Bacillus* sp. 2 + carbon and nitrogen source and *Bacillus* sp. 2 + carbon and nitrogen source resulted in a reduction of 86.71 and 82.73%, respectively (see Fig. 6).

Characteristics of Biologically Treated Effluent

In the dye effluent treated by bacterial strains (*Bacillus* sp. 1 and 2), the pH, EC, biologic oxygen demand (BOD), chemical oxygen demand (COD), TSS, and TDS were reduced moderately. The pH was 7.32 and EC was 8.61 dS/m. The TSS, TDS, BOD, and COD were 3879, 3927, 529, and 1916 mg/L, respectively.

Conclusion

Incubation experiments with bacterial strains and their mutants having different inoculum concentrations and incubation periods along with carbon and nitrogen sources were tested for their decolorization potential. The bacterial strains were optimized for higher growth and biomass production with different carbon and nitrogen sources at various pH values and temperatures. The mutant strains of bacteria grew faster and reduced the color of the dye effluent comparatively greater than the wild isolated strains. This work could serve as an important base for developing economic treatment for providing reusable clean water for industrial use.

References

1. Zollinger, H. (1987), in *Color Chemistry—Synthesis, Properties and Applications of Organic Dyes and Pigments,* VCH, New York, pp. 92–100.
2. Pierce, J. (1994), *J. Soc. Dyers Colorists* **110,** 131–134.
3. Nigam, P., Banat, I. M., Singh, D., and Marchant, R. (1996), *Process Biochem.* **31,** 435–442.
4. Yatome, C., Ogawa, T., Hayashi, H., and Ogawa, T. (1991), *J. Environ. Sci. Health A* **26,** 471–485.
5. Zhou, W. and Zimmermann, W. (1993), *FEMS Microbiol. Lett*. **107,** 157–162.
6. Pandey, A., Nigam, P., Soccol, C. R., Soccol, V. T., Singh, D., and Mohan, R. K. (2000), *Biotechnol. Appl. Biochem.* **31,** 135–152.
7. Dorinadince, L., Fureoane, S., Vamanu, E., Mirela burcea H and Campeanu (2001), *Roum Biotechnol. Lett.* **6,** 111–118.
8. Ogawa, T., Yatome, C., Idaka, E., and Kamiya, H. (1986), *J. Soc. Dyers Colorists* **2,** 12–14.
9. Wuhrmann, K., Mechsher, K., and Kappeler, T. (1980), *Eur. J. Appl. Microbiol. Biotechnol*. **9,** 325–338.
10. Verma, S. R., Tyagi, A. K., and Daleta, R. C. (1974), *Indian J. Environ. Health* **19,** 165–175.

11. Mou, O. G, Lim, K. K., and Shen, H. P. (1991), *Biotechnol. Adv*. **9,** 613–622.
12. Murali, S., Narayan, A., Vidyavati, E., and Sriniketan, G. (2000), *J. Indian Pollut. Control* **16,** 211–215.
13. Yatome, C., Ogawa, T., Hishida, H., and Tagochi, T. (1990), *J. Soc. Dyers Colorists* **106,** 280–282.
14. Hu, T. L. (1994), *Bioresour. Technol*. **49,** 47–51.
15. Waksman, S. A. and Fred, B. (1922), *Soil Sci.* **14,** 27–28.
16. Seelay, J. and Vandemark, A. J. (1994), *J. Bacteriology* **176,** 656–664.
17. Clowes, R. C. and Hayes, W. (1968), *Experiments in Microbial Genetics*. Blackwell, Edinburgh, UK.