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Study on the Microbial Degradation Tannic Acid by Alcaligenes species

Vasukidevi Ramachandran*

Department of Biomedical Engineering, Bharath University, Chennai-600073, Tamil Nadu, India

***Corresponding author:**
Vasukidevi Ramachandran

vasukideviram@gmail.com

Associate Professor, Department of Biomedical Engineering, Bharath University, 173, Agaram Road, Selaiyur, Chennai-600073, Tamil Nadu, India.

Tel: +91 99 4034 6061

Abstract

The objective of this research was to identify the bacteria responsible for the degradation of tannery effluent. The tannery effluent samples were collected from the outlets of a leather industry, which is situated near Dindigul in Madurai district of Tamil Nadu, India. The microorganisms that were present in the tannery effluent were isolated by using dilution plate method. The bacteria which were able to utilised tannic acid as sole carbon source was isolated by using tannic acid amended minimal medium. The results of morphological, physiological & biochemical tests led to the identification of the bacteria to be *Alcaligenes* spp. This bacterium degraded tannic acid very effectively when compared to other isolated organisms. The standard graphs for tannic acid were drawn and the results were tabulated. The effect on growth and degradation of tannic acid of *Alcaligenes* spp. by changing different parameters were studied.

Keywords: Tannic acid; Alcaligenes species; Tannery effluents; Microbial degradation

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Introduction

India occupies a predominant position in the world production of leather from animal skins. Even though the growing economy is increasingly supported by leather industries, it should be realised that this achievement is being made at the sacrifice of arable land, quality of drinking water and human health.

There are about 450 tanneries in the state and most of them are located in North Arcot, Madurai, Dindigul, Salem, and Chengalpattu districts, Tamil Nadu, India [1]. Although the tannin industry has been in existence in the country for a long time, the problems caused are viewed seriously only in recent years. With the establishment of an export potential for our leather products the number of tanneries are steadily increasing. Most of the tanneries have come up in an unplanned manner without consideration for satisfactory disposal of the effluents [2]. This has resulted in serious water pollution and obnoxious conditions in regions where tanneries are located [3].

Tannery effluent is one of the most complex and potential pollutants in the area. The main polluting constituents of the effluents are chloride, sulphide, sodium, chromium, dissolved solids, suspended organic matter and tannin. They are responsible for the high Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) of effluent.

Discharged of untreated tannery effluents in to streams depletes the dissolved oxygen of the stream, destroys the aquatic life and renders the streams unsuitable for community water supply and for other beneficial uses [4]. The suspended matter present in the effluents form sludge banks on the stream bed and cause obnoxious odours. Discharge of tannery effluents on land pollutes ground water with high chlorides and chromates. The productivity of the soil decreases when tannery effluents are applied on fields indiscriminately and in course of time the land becomes unfit for agriculture [3].

Medical cases have been reported where anthrax was transmitted to bathers and water cress gatherers through contact with tannery effluents discharged in water courses. It has been reported that arsenic and chromium present in tannery effluent have rendered drinking water unsuitable. Discharge of untreated effluents on land has also resulted in heavy chloride pollution of well water. There is an urgent need to find a proper solution to this problem [3].

The Pollution Control Board (Government of Tamil Nadu) insists the tanners to regulate the quality of effluent discharged by the tanneries, but most of the tanneries are reluctant to install treatment plants and continue to discharge untreated effluent due to cost and maintenance [5-9]. Nevertheless, because of the high pollution load, the tannery effluent needs effective treatment before being disposed on to land or to any water body.

During recent years, several methods have been proposed for the treatment of the effluents. Some of them are primary treatment in fill and draw tanks [5], biodegradation of wastes using microbes, chemical treatment [2] and treatment with the aquatic weed, Eichhornia [10].

In India, the scientists at the National Environmental Engineering Research Institute, Nagpur, College of Engineering, Guindy, Chennai, Central Leather Research Institute, Adyar, Chennai and Tamil Nadu Agricultural University, Coimbatore have assessed the pollution potential of some of the industrial effluents. Attempts are being made to treat the sewage and other waste to utilise the effluent for agricultural purposes. An appropriate and cost effective treatment method for tannery effluent to make it suitable for irrigation for agricultural land is urgently required. Biodegradation technology is one of the recent methods most successfully applied in the treatment of several kinds of industrial wastes. Biological treatment methods are recognised as the most desirable, environmental friendly easiest and cost effective method in pollution abatement programmes. Moreover, it is free from sludge accumulation which is a problem for disposal as in the case of chemical treatment.

Tannery effluent contains large amounts of wastes, especially tannins. Tannins are defined as water soluble, phenolic compounds, with molecular weight ranging from 500 to 3000 mg. The essential property of tannins is their ability to combine with protein, cellulose, gelatine as well as with pectin. Tannins are classified in to distinct groups condensed or non-hydrolysable tannins and hydrolysable tannins. Esters of sugars and phenolic acids or their derivatives are referred to as hydrolysable tannins. The hydrolysable tannins are again subdivided into gallo tannins and ellagitannins. Gallo tannins on hydrolysis yield glucose and Gallic acid [11]. On hydrolysis, ellagitannin yield Gallic acid and the derivatives, especially, ellagic acid.

Toxicity of tannery effluent is due to the presence of high concentrations of tannins. Tannins are of plant origins and are used for tanning of skin and hide. Unused tannins present in the effluent have toxic effects on the protoplasm of living cells of animals, plants and microorganisms [12,13]. Tannins adversely affect not only the yield of crops but also are detrimental to the useful microorganisms such as Azotobacter and Rhizobium [14,15].

In recent years, there is a growing interest in the potential use of microorganisms for tannin degradation. Degradation of tannic acid and tannins by fungi and yeast has been well documented [7]. But, fungi are slow degraders and cause atmospheric pollution through their spores. Bacteria are considered highly sensitive to tannins but some isolates survive and even degrade tannins [9].

Tannic acid hydrolysable tannin) is bacteriostatic and considered to be resistant to bacterial attack. However *Staphylococcus* sp and *Bacillus* sppare reported to utilise tannic acid [16,17]. Fungi like are *Aspergillus niger, A. flavus, Aspergillus* spp., *Penicillium* spp. and *Rhizopus* spp. are also isolated from the tannery wastes.

Our objectives are to isolate and identify the microorganisms present in the tannery wastes and to evaluate the efficacy of the microorganisms to degrade tannic acid by changing different parameters

Materials and Methods

Tannery effluents were collected from the outlet of a leather industry, which is situated near Dindigul, in Chempatti district of Tamil Nadu, India. The effluent samples were collected in sterile bottles and was transported to the laboratory in a cool box and kept refrigerated till processed. All the bacteriological analyses were done within 24 h. Many microorganisms were isolated from the tannery effluent and the bacterial isolate *Alcaligenes* spp. was used to study biodegradation of tannin. Certain biochemical tests were performed for the confirmation of the bacterial isolate.

Microbial population in the tannery wastes, samples were collected and were analysed by dilution plate method. All the aromatic substances were sterilised by Millipore filtration with 0.45 µ pore filter.

Number of microorganisms that were isolated from the tannery wastes and the isolates were selected for tannic acid degradation from the tannic acid amended minimal medium.

Morphological studies shape size, surface, elevation, edge (in nutrient and minimal agar media) were studied. Motility was studied employing Hanging drop method.

Gram staining was performed in order to determine whether it is gram negative or gram positive. The culture was taken and made smear on the slide. The primary stain, Crystal violet was stained for 1 min, which resulted in purple colour. Gram's Iodine, which is used as a mordant, was applied to cell for 1 min. The function of their mordant here is to combine with crystal violet to form a relatively insoluble compound only in gram positive bacteria, but not in the gram negative bacteria. Then 95% ethanol, was added, which is used as a decolourising agent, for 30 s. It was quickly washed and a counter stain, safranin was added for 30 s. Biochemical tests for the production of indole was detected by adding Kovac's reagent, which produce a cherry red colour reagent for a positive result. No colour change indicates the negative result.

To determine the ability of microorganisms to degrade the amino acid tryptophan in the medium. The medium used is Sim medium. The methyl red test was done to determine the ability of microorganisms to oxidise glucose with the production and stabilisation of high concentration of acid as an end product. The MR-VP medium is used for this test. The production of acid is detected by adding methyl red. The red colour indicates the positive result. Voges-Proskauer test is to determine the production of acetyl-methyl carbinol from the acids produced from the glucose metabolism. This test is detected by adding by Barrett's reagent. The positive result gives a deep rose to crimson colour. Citrate utilisation test was done to determine the ability of ferment citrate as main carbon source present on the medium.

The bromo thymol blue colour change to deep blue colour for the positive result. The medium used is the Simmons citrate medium.

This Hydrogen Sulphide production test to determine the ability

of microorganisms to produce hydrogen sulphide from sulphur containing amino acids and inorganic sulphur compounds was done. The positive result develops a black colour in the medium. This Carbohydrate Fermentation test is to determine the ability of microorganisms to degrade and utilise carbohydrates present in the medium with the production of gas and acid was also carried out. Starch hydrolysis test was done to determine the ability of microorganisms to produce enzymes to degrade starch present in the medium. A single line streak was made and zone of lysis was detected by flooding with iodine solution. A clear zone indicates the positive result. Urease activity was studied by growing the cultural overnight in the Urease medium. The positive result gives a pink colour to the medium. Catalase test to determine the ability to produce catalase was carried out. Test for reduction of nitrate was carried out by inoculating the bacteria culture in nutrient broth containing 0.1% potassium nitrate. After incubation, a pinch of zinc dust was added Pink colour indicated the absence of nitrate reductase activity and failure of colour development revealed that nitrate was completely reduced.

Concentration of tannin in the effluent was quantified by using the protein precipitation method.

Tannin acid solution (0.1 ml) was made up to 1 ml with distilled water. To this 2 ml of bovine serum albumin (1 mg/ml acetate buffer, pH 4.8) was added, mixed thoroughly and incubated for 15-30 min at room temperature. Then supernatant was discarded. The pellet and walls of the tubes were washed with 0.2 M acetate buffer. The precipitate was dissolved in 4ml of SDS-TEA solution (1% SDS 5% Triethanolamine solution). To each tube 1 ml of 0.01 M ferric chloride was added and mixed immediately. After 15-30 min, the red colour was measured at 510nm. The blank contains 1 ml of ferric chloride, 1 ml buffer and 4 ml of SDS-TEA.

Effect of different concentration of tannic acid on growth was studied by sterilised tannic acid which was aseptically amended to the minimal medium to get concentrations ranging from 0.25-2.0% as sole carbon source. Minimal medium without tannic acid and with glucose was treated as a control. The culture was inoculated and incubated at 30° on a rotating shaker at 150 rpm. Aliquots of 5 ml culture were withdrawn at 12 h interval for 72 h and growth was measured at 510 nm.

The effect of pH on the growth and degradation of tannic acid, Effect of temperature on growth and degradation of tannic acid and Effect of co-substrates on growth and degradation of tannic acid were also observed.

Results and Discussion

Standard graph for tannic acid

The standard graphs for tannic acid observed are shown in the **Figures 1 to 10**. The results are tabulated in **Tables 1-10.**

Characteristics of tannic acid degradation bacterium were observed with ability to survive and grown in high concentrations of tannic acid. They bacterium were coccal rods when observed under the microscope. They were gram negative bacteria and motility was by peritrichous flagella. Growth characteristics show that there were obligate aerobe and the temperature for optimal growth was 20-37°C.

In biochemical observations they were oxidase and catalase positive.

Figure 1 Tannery effluents discharged in to a water body.

Figure 2 Tannery effluents discharged in open land.

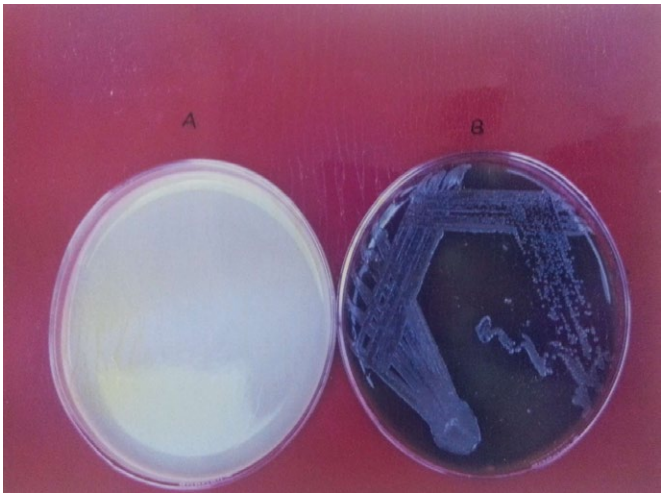

Figure 3 Colonies of *Alcaligenes* spp. in minimal plates with tannic acid.

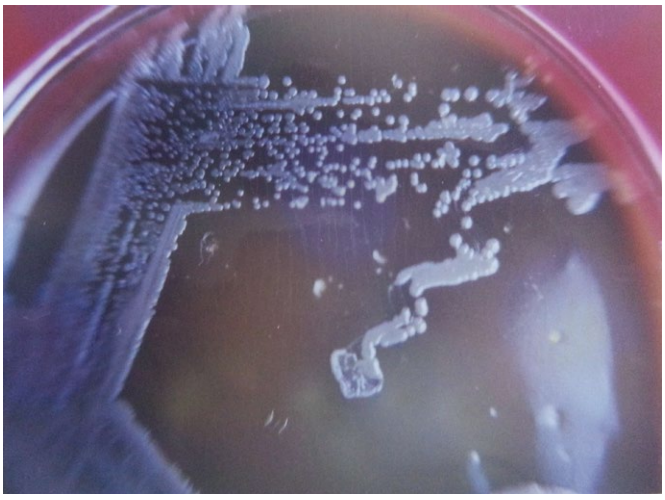

Figure 4 Isolated colonies of *Alcaligenes* spp.in minimal plates.

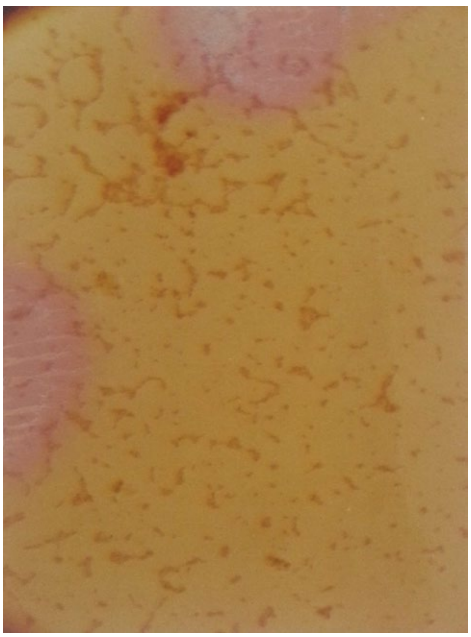

Figure 5 Microscopic view of *Alcaligenes* spp.

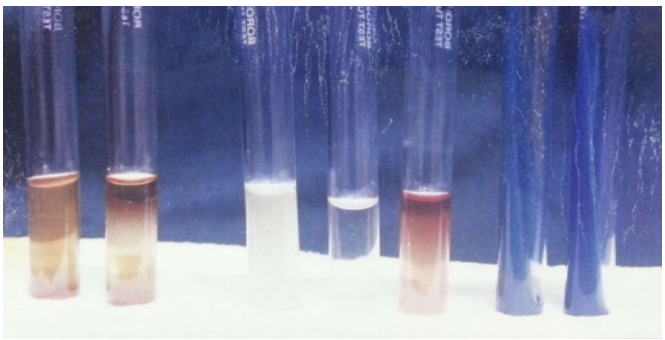

Figure 6 IMViC.

Figure 7 Sugar fermentation test-glucose.

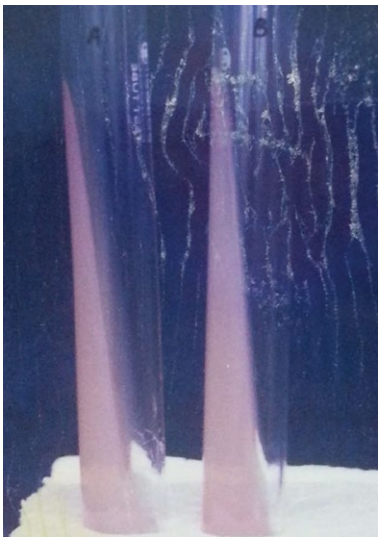

Figure 8 Urease test.

Figure 9 Catalase test.

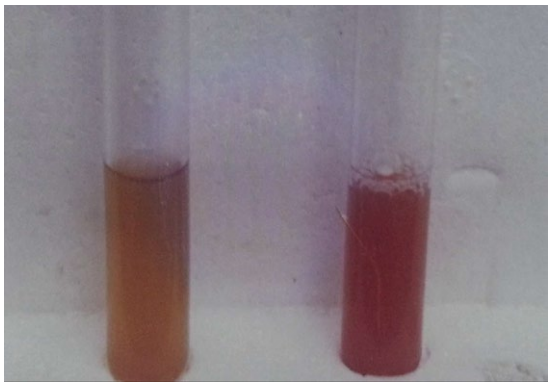

Figure 10 Nitrate reductase test.

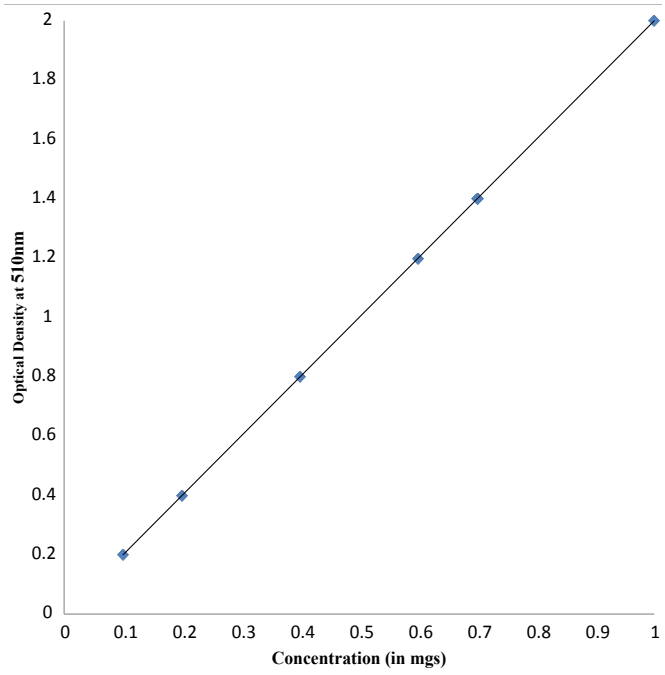

Figure 11 Standard Graph for tannic acid.

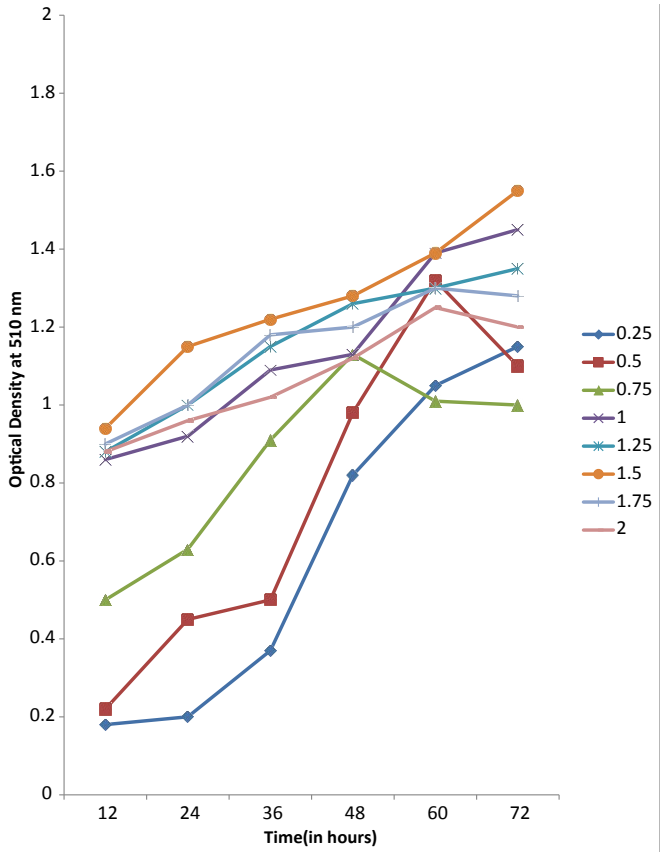

Figure 12 Effect of different concentration of tannic acid on the growth of *Alcaligenes* spp.

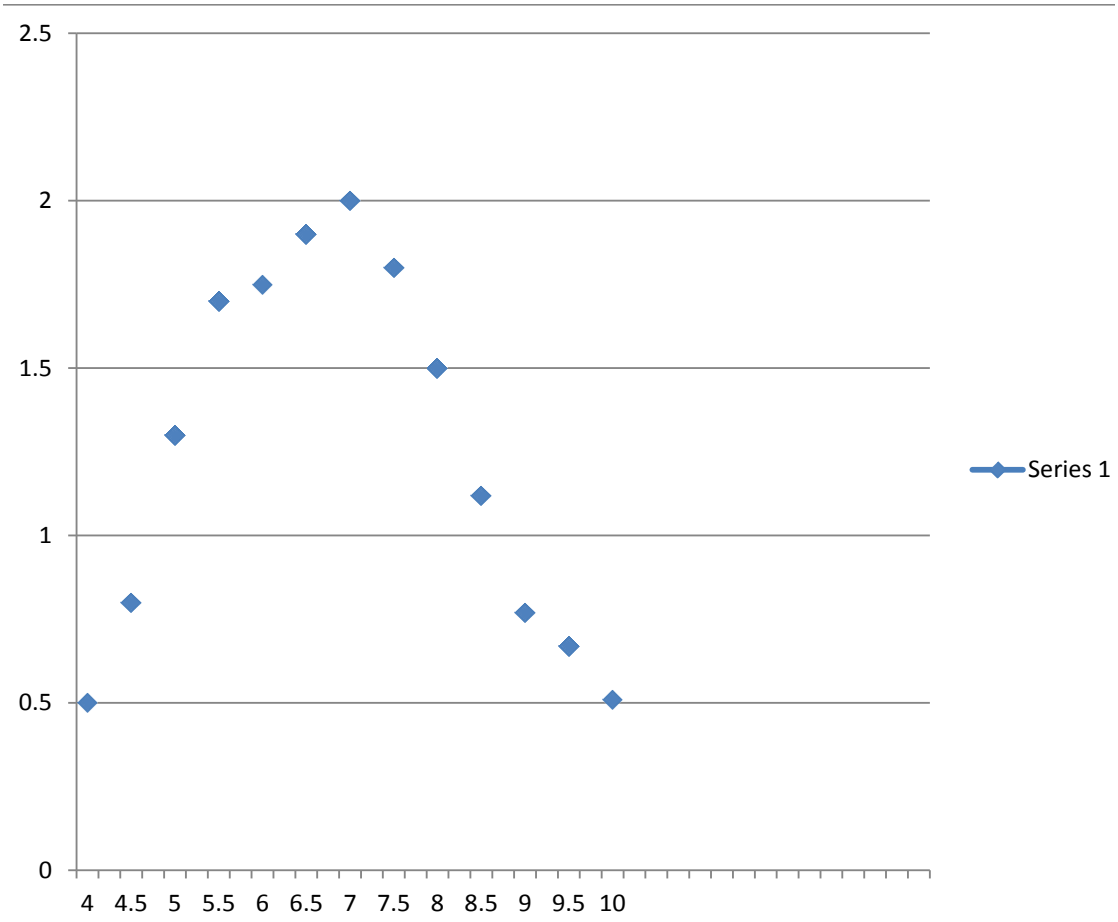

Figure 13 Effect of pH on the growth of *Alcaligenes* spp.

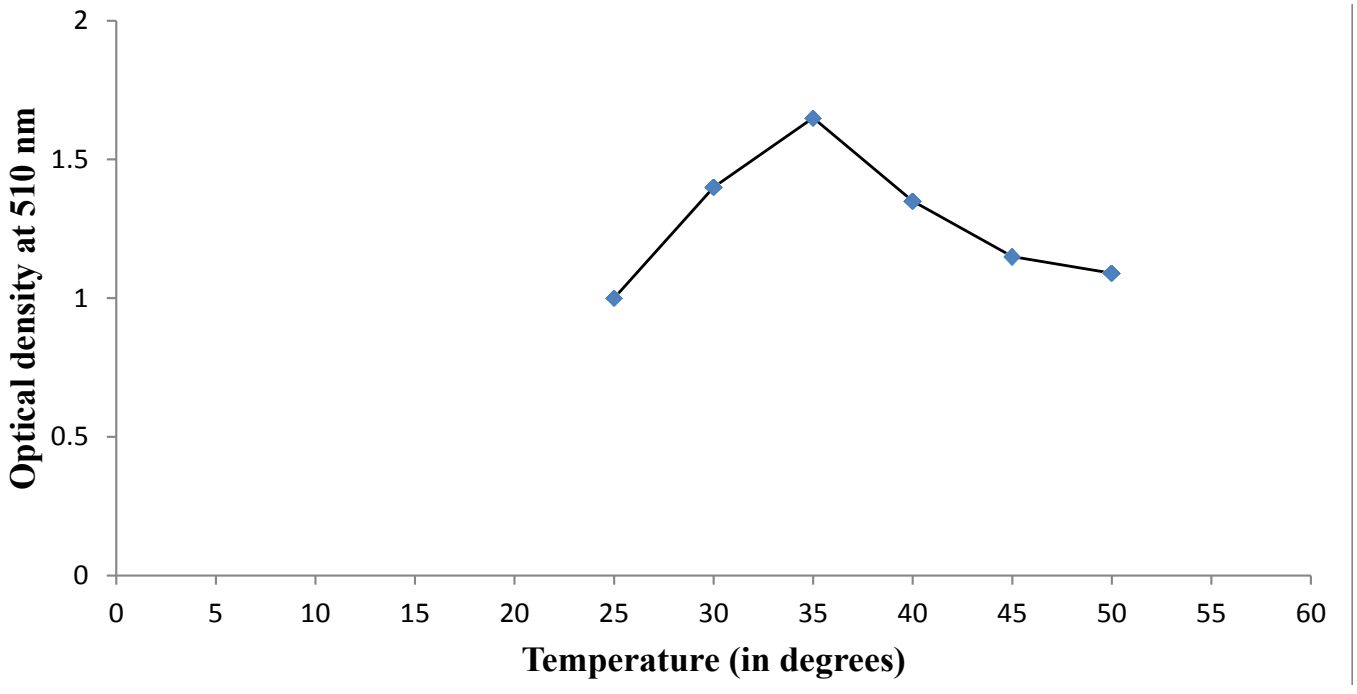

Figure 14 Effect of different temperature growth of *Alcaligenes* spp.

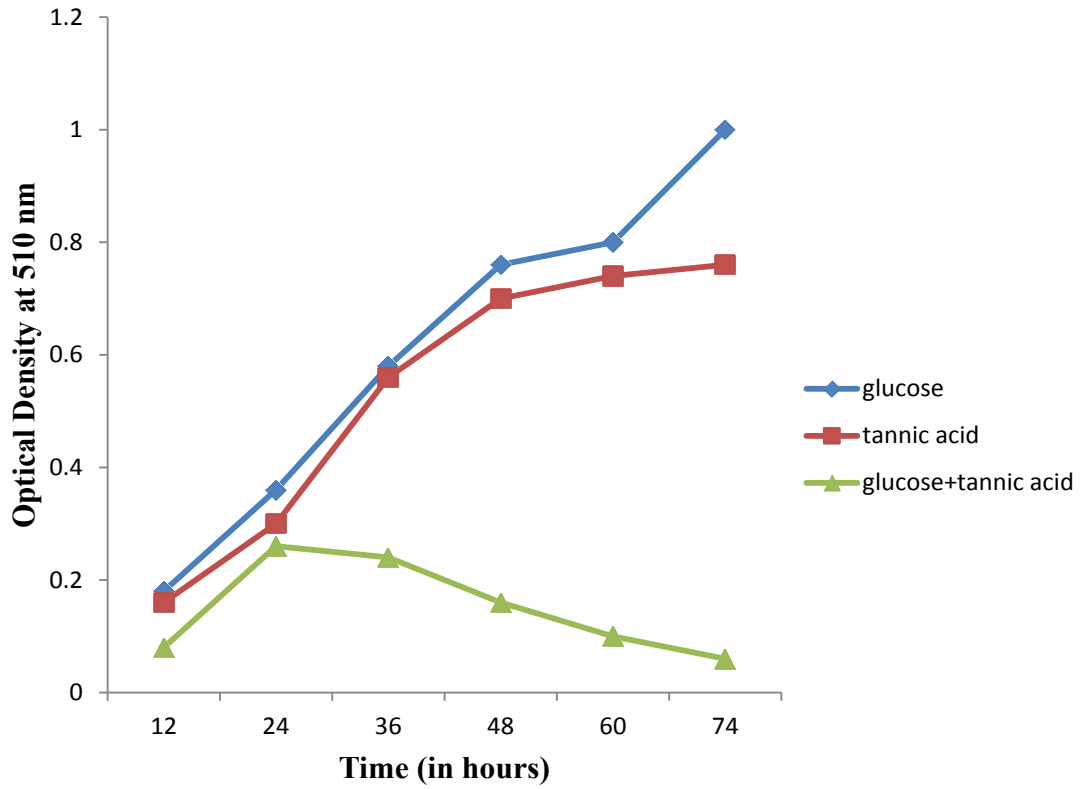

Figure 15 Effect of glucose on the growth of *Alcaligenes* spp.

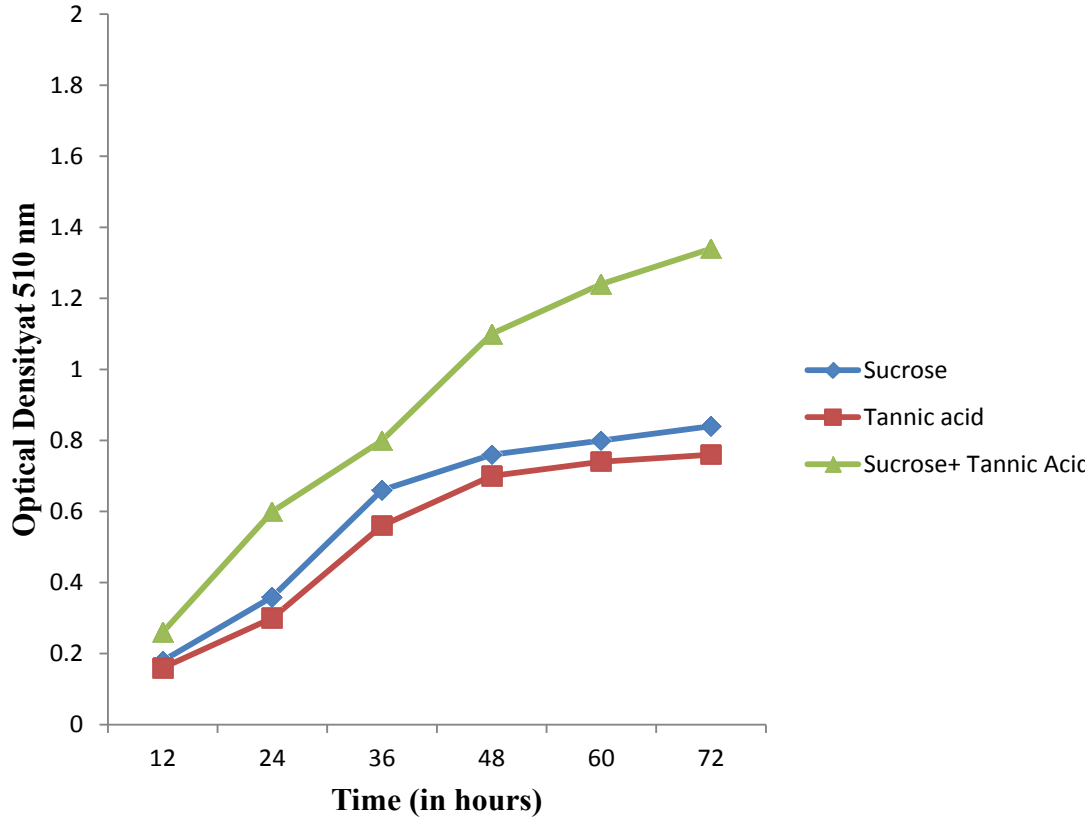

Figure 16 Effect of sucrose on the growth of *Alcaligenes* spp.

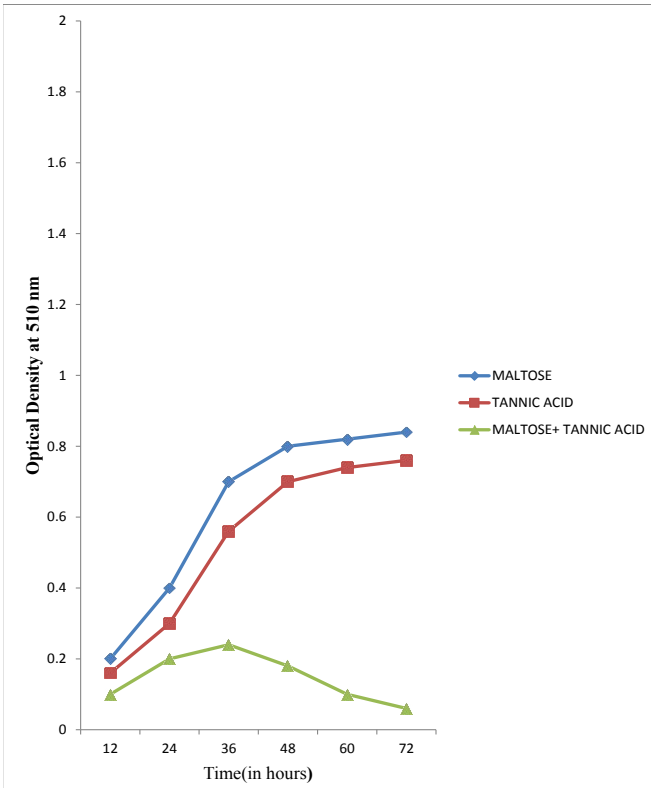

Figure 17 Effect of maltose on the growth of *Alcaligenes* spp.

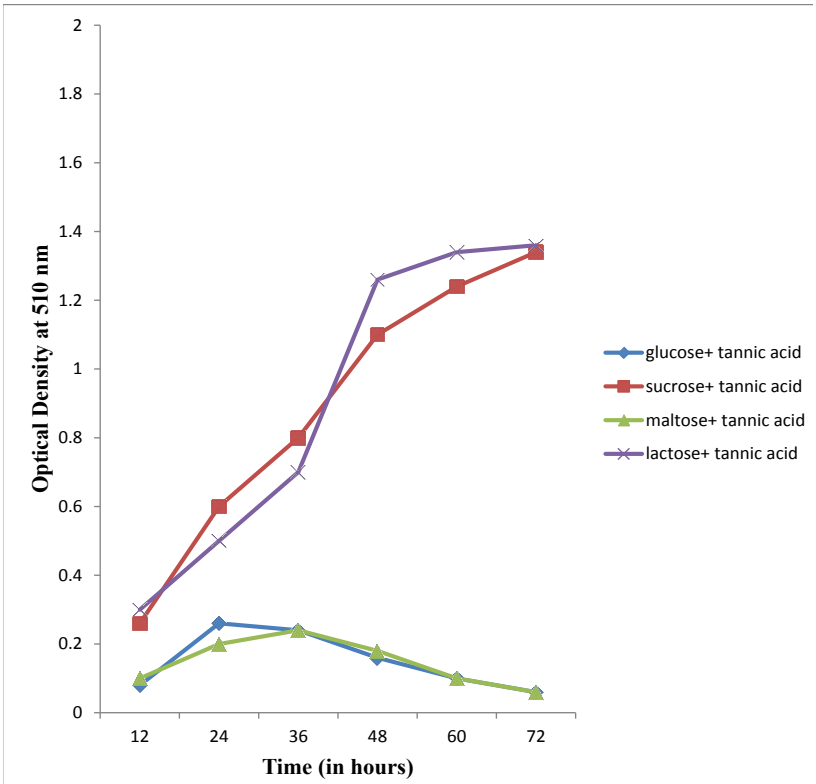

Figure 18 Comparison of the effects of different co-substrates with tannic acid on the growth of *Alcaligenes* spp.

Table 1 Standard graph for tannic acid.

Concentration (in mg)	Optical Density at 510 nm
0.1	0.2
0.2	0.4
0.3	0.64
0.4	0.8
0.5	0.96
0.6	1.2
0.7	1.4
0.8	1.64
0.9	1.84
1.0	2.0

Table 2 Biochemical tests.

Tests	Results
Indole Production	Negative
Methyl Red	Negative
Voges-Proskauer	Positive
Citrate Utilisation	Positive
Hydrogen Sulphide production	Negative
Carbohydrate Fermentation (Glucose) (Sucrose)	 Negative Negative
Starch Hydrolysis	Negative
Gelatin Hydrolysis	Negative
Urea Hydrolysis	Negative
Oxidase Activity	Positive
Nitrate Reductase	Positive
Catalase Activity	Positive

Table 3 Effect of pH on the growth of *Alcaligenes* spp.

pH	Optical density at 510 nm
4.0	0.5
4.5	0.8
5.0	1.3
5.5	1.7
6.0	1.75
6.5	1.9
7.0	2.0
7.5	1.8
8.0	1.5
8.5	1.12
9.0	0.77
9.5	0.67
10.0	0.51

Table 4 Effect of different concentrations of tannic acid on the growth of *Alcaligenes* spp.

Time (in h)	Concentrations (in percentage)							
	0.25	**0.5**	**0.75**	**1.0**	**1.25**	**1.5**	**1.75**	**2.0**
12	0.18	0.22	0.50	0.86	0.88	0.94	0.90	0.88
24	0.20	0.45	0.63	0.92	1.0	1.15	1.0	0.96
36	0.37	0.50	0.91	1.09	1.15	1.22	1.18	1.02
48	0.82	0.98	1.13	1.13	1.26	1.28	1.20	1.12
60	1.05	1.32	1.01	1.39	1.30	1.39	1.30	1.25
72	1.15	1.10	1.0	1.45	1.35	1.55	1.28	1.20

Table 5 Effect of pH on growth and degradation of tannic acid.

Sl. No.	pH	Time interval					
		24 h		48 h		72 h	
		Optical Density At 510 nm	Residual Tannin (in mg)	Optical density At 510 nm	Residual Tannin (in mg)	Optical density At 510 nm	Residual Tannin (in mg)
1	5.0	1.86	0.93	1.7	0.85	1.4	0.7
2	5.5	1.7	0.85	1.6	0.8	1.2	0.6
3	6.0	1.2	0.6	1.0	0.5	0.7	0.35
4	6.5	0.8	0.4	0.6	0.3	0.4	0.2
5	7.0	0.6	0.3	0.5	0.25	0.3	0.15

Table 6 Effect of different temperatures on the growth of *Alcaligene* spp.

Temperature (in degrees)	Optical Density at 510 nm
25	1.0
30	1.4
35	1.65
40	1.35
45	1.15
50	1.09

Table 7 Effect of temperature on growth and degradation of tannic acid.

S. no.	Temperature (in degrees)	Time Interval					
		24 h		48 h		72 h	
		Optical Density At 510 nm	Residual Tannin (in mg)	Optical density At 510 nm	Residual Tannin (in mg)	Optical Density At 510 nm	Residual Tannin (in mg)
1	25	1.6	0.8	1.4	0.7	1.2	0.6
2	30	1.2	0.6	1.1	0.55	0.96	0.48
3	35	1.0	0.5	0.8	0.4	0.5	0.25
4	40	1.16	0.58	1.0	0.5	0.8	0.4
5	45	1.2	0.6	1.12	0.56	1.0	0.5
6	50	1.5	0.75	1.4	0.7	1.2	0.6

Table 7a Effect of glucose on the growth of *Alcaligenes* spp.

Time (in h)	Optical Density at 510 nm		
	Glucose	Tannic Acid	Glucose+Tannic acid
12	0.18	0.16	0.08
24	0.36	0.3	0.26
36	0.58	0.56	0.24
48	0.76	0.7	0.16
60	0.8	0.74	0.1
72	1.0	0.76	0.06

Table 7b Effect of sucrose on the growth of *Alcaligenes* spp.

Time (in h)	Optical Density at 510 nm		
	Sucrose	Tannic acid	Sucrose+Tannic acid
12	0.18	0.16	0.26
24	0.36	0.3	0.6
36	0.66	0.56	0.8
48	0.76	0.7	1.1
60	0.8	0.74	1.24
72	0.84	0.76	1.34

Table 8 Effect of maltose on the growth of *Alcaligenes* spp.

Time(in h)	Optical Density at 510 nm		
	Maltose	Tannic Acid	Maltose+tannic acid
12	0.2	0.16	0.1
24	0.4	0.3	0.2
36	0.7	0.56	0.24
48	0.8	0.7	0.18
60	0.82	0.74	0.1
72	0.84	0.76	0.06

Table 9 Effect of lactose on the growth of *Alcaligene* spp.

Time (in h)	Optical density		
	Lactose	Tannic Acid	Lactose+ tannic acid
12	0.2	0.16	0.3
24	0.38	0.3	0.5
36	0.6	0.56	0.7
48	0.8	0.7	1.26
60	0.86	0.74	1.34
72	0.9	0.76	1.36

Table 10 Comparison of the effects of different co-substrates with tannic acid on the growth of *Alcaligene* spp.

Time (in h)	Optical Density at 510 nm			
	Glucose+Tannic Acid	Sucrose+Tannic Acid	Maltose+Tannic Acid	Lactose+Tannic Acid
12	0.08	0.26	0.1	0.3
24	0.26	0.6	0.2	0.5
36	0.24	0.8	0.24	0.7
48	0.16	1.1	0.18	1.26
60	0.1	1.24	0.1	1.34
72	0.06	1.34	0.06	1.36

Table 11 Effect of co-substrates on growth and degradation of tannic acid.

Sl. No.	Co substrates	Time interval					
		24 h		48 h		72 h	
		Optical Density at 510 nm	Residual Tannin (in mg)	Optical Density at 510 nm	Residual Tannin (in mg)	Optical Density at 510 nm	Residual Tannin (in mg)
1	Glucose	1.8	0.9	1.1	0.55	1.0	0.5
2	Sucrose	1.7	0.85	1.1	0.55	0.9	0.45
3	Maltose	1.79	0.89	1.0	0.5	0.9	0.45
4	Lactose	1.5	0.75	0.5	0.25	0.2	0.10

They were non-pigmented and Indole was not produced. Citrate was utilised as sole carbon source. They were methyl red negative and Voges-Proskauertest positive. Carbohydrates were not usually utilised. They did not produce hydrogen sulphide and they reduce nitrates. They were urease negative and did not hydrolyse starch and gelatin. The results for biochemical tests were tabulated in the **Table 2.**

From the microscopic examination, cultural and biochemical reactions, the organism was identified as *Alcaligenes* spp., which degrade tannic acid effectively degradation of tannic acid by *Alcaligenes* spp.

Conclusion

The effect of various concentration of tannic acid on growth of the bacterium was studied. The various concentrations of tannic acid used were 0.25%, 0.5%, 0.75%, 1.0%, 1.25%, 1.5%, 1.75% and 2.0%. The growth of the bacterium increased with the increasing concentration of tannic acid. Maximum growth was observed at 1.5% concentration. After 1.5% of concentration, the growth of the bacterium decreased. So the optimum concentration of tannic acid was considered as 1.0%. The effect of growth on tannic acid degrading bacterium was shown in the **Figure 2** and the results were tabulated in the **Table 3.**

Minimal medium containing 1% tannic acid and 1% sugar (glucose, sucrose, maltose, lactose) solution were prepared. The medium was inoculated and incubated at 30°C for 72 h on a rotator shaker. The growth was measured at 510 nm and the residual tannin was estimated at 24 h interval.

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