JOURNAL OF PURE AND APPLIED MICROBIOLOGY, April 2007. Vol. **1**(1), p. 21-28

Production of Gallic Acid from Tannin using Three Different Fungal Strains by Submerged Fermentation

N. Lokeswari[1]*, K. Jaya Raju[2] and M. Narasimha Rao[2]

[1]Center for Biotechnology, Department of Chemical Engineering,
Andhra University, Visakhapatnam - 530 003. India.
[2]Faculty of Chemical Engineering, Department of Chemical Engineering,
Andhra University, Visakhapatnam - 530 003. India.

(Received: 03 February 2007; accepted: 11 March 2007)

Hydrolysis of Gallo tannin to Gallic acid using three tannase producing fungal strains were *Aspergillus niger* MTCC 282, *Fusarium Solani* MTCC 350 and *Trichoderma viride* MTCC 167 by submerged fermentation from myrobalan. These Fungal mycelia preinduced with 5g L^{-1} Gallo tannin, adjusting pH to 6 with ammonium hydroxide (10%). Maximum hydrolysis of Gallo tannin was obtained by fungal mycelia of *Fusarium solani* and *Trichoderma viride* at 35°C and 45°C after 180 and 60 min of residence period respectively. Optimum substrate concentration required for maximum hydrolysis was 20 g L^{-1} at pH 5 for both the fungi. Various parameters like substrate, pH, temperature, inoculum fermentation time were optimized for hydrolysis of Gallo tannin to gallic acid from the above said three different strains which produces tannase enzyme when induced with 5g L^{-1} Gallo tannin by submerged fermentation.

This work aims at finding a suitable fungi source of tannase and development of a process for microbial hydrolysis of Gallo tannins to yield gallic acid, which serves as a starting material for the manufacture of widely used antifolic antibacterial drug trimethoprim.

Keywords: Gallo tannin, Gallic acid, Submerged Fermentation, Tannase, *Fusarium solani, Trichoderma viride, Aspergillus niger.*

Gallic acid (3, 4, 5-Tri hydroxyl benzoic acid) is a versatile precursor used for the manufacture of a variety of chemicals used in food and pharmaceutical industries.

Tieghem (1867) carried out classical studies in connection with the gallic acid fermentation. Gallic acid, the product of tannin hydrolysis finds application in many fields including dye – making, pharmaceutical, leather, food industry and chemical industries (Hadi *et al.*, (1994); Lekha and Lonsane, (1997); Mukherjee and Banerjee, 2003). Tannins are esters of gallic acid and are obtained from plant galls, fruits pods and leaves, Oak bark, horse chest nut, etc. Nishizawa *et al.* (1983).

Haslam (1983) reported that tannins may occur either in isolated individual cells, in groups or chains of cells or in special cavities or sacs. The young, actively growing issue of plants is also liable to be very rich in tannins. The present study has been taken up with a view of exploring the possibilities of using myrobalan as a substrate for fermentation by tannase producing microorganism resulting in the biotransformation of myrobalan tannin to Gallic acid. At present, India has to import to meet most of its Gallic acid requirement myrobalan (*Terminalia chebula*) is abundantly available in India. Myrobalan is a potential raw material for the production of gallic

* To whom all correspondence should be addressed.
Mobile: +91-9290245940; Fax: +91-891-2551987;
E-mail: lokeswarin@hotmail.com.

acid. Thus, for the present study, it has been taken up as a substrate for fermentation by tannase producing microorganisms. Tannase (tannin acyl hydrolase, E C 3.1.1.20), an inducible extra – cellular enzyme produced by a number of animals, plants and microbes. It has wide application in tannery, alcohol industry, pharmaceuticals and beverage industries. It is also responsible for the bioconversion of hydrolysable tannins to gallic acid (Lekha and Lonsane, 1997; Mukharjee and Banerjee, 2003). Microbial tannase is more stable than tannase from other sources like plants or animals.

Kudson (1913), reported that the major contribution to Gallo tannin fermentation was demonstrated the toxic nature of tannic acid to most of the fungi, at low concentration except various others species of *Aspergillus* and *Penicillium*. Haslam *et al.,* (1961) first purified tannase from *A. niger* by Ion-exchange chromatography. Haslam (1966) resolved tannase into two separate enzymes, an esterase and dipeptidase with specificity's to methyl gallate and m-digallic acid respectively. Purification of tannase from *Aspergillus oryzae* (Iibuchi *et al.,* 1968), *Candida* sp (Aokik *et al.* 1976) and *A. niger* (Barthomuf *et al.* 1994) has been reported. The biochemical properties of tannase enzyme were investigated by Barthomuf *et al.* (1994).

Rajakumar and Nandy (1983); Kawakudo *et al.*, (1991); reported that the tannase is produced by *Penicillium* and also by bacteria (Deschamp *et al.*, 1983) and yeast (Aokik *et al.*, 1976). The major commercial applications of tannase are in the manufacture of instant tea or acorn wine and the production of gallic acid (Chae and Yu, 1983; Pourrat *et al.*, 1985). *Aspergillus niger* MTCC 282, *Fusarium solani* MTCC 350 and *Trichoderma viride* MTCC 167, the fungi recently reported to produce tannase enzyme when induced with Gallo tannin present in the incubation medium. (Bajpai and Patill, 1997).

The literature survey indicates that there are hardly 60 number of tannase producing microorganisms available. Out of which, we selected three different strains namely *A. niger*, *F. solani* and *T. viride* for the present work.

Microbes are dependent on the affecting process parameters for their growth, and product yield and in term depend on the enzyme synthesis by the microbe. In the present study the effects of the process parameters on tannase and gallic acid production by submerged fermentation were studied and their optimum levels were determined.

The present work proposes to screen strains for economic source of tannase and to develop a process for the production of gallic acid using a spectrophotometric method. For the present work, three strains of filamentous fungi were obtained from MTCC.

MATERIALS AND METHODS

Microorganisms and growth

Fungal strains of *A. niger* (MTCC167), *F. solani* (MTCC 350) and *T. viride* (MTCC160) were procured from microbial type culture collection, Chandigarh, India. These fungi were grown on 0.01% Gallo tannin supplemented Czapek yeast extract agar slants for *A. niger*, potato sucrose agar slants for *F. solani* and potato dextrose agar slants for *T. viride* and maintained at 4°C. The slants were sub-cultured routinely at an interval of 4-5 weeks.

Raw Materials

Myrobalan (*Terminalia chebula*) obtained from Girijan Co. Ltd. Visakhapatnam.

Extraction of tannins

Myrobalan fruits powder were dried and milled to get the particle size below 5 mm, Tannins were extracted by pressure autoclave at 10 PSI for half an hour. After 30 min the extract was filtered through cloth. This extract was evaporated and the obtained powder form was used for the entire experiment.

Estimation of total tannins

The estimation of tannin content was done following the protein precipitation method of Hagerman and Butler (1978). Bovine serum albumin (BSA) was taken as the standard protein.

Preparation of spore suspension

8 ml of sterile distilled water was taken in 50 ml conical flask. The mycelia of the slant cultures were scraped off in 2 ml of distilled water.

The resulting spore suspension was mixed to obtain a uniform suspension. This spore suspension was then added to distilled water to give 8 ml of distilled water to give 10 ml of spore suspension.

Preparation of induced inoculum

Tannase being an adaptive enzyme, preinduced inoculums is required to be prepared. The medium used for growing fungi, *A. niger, F. solani* and *T. viride* were grown in potato dextrose broth containing Gallo tannin 5 g/lit adjusted to pH 5.6. 50 ml of above said medium was taken in 100 ml of conical flasks. It was then sterilized and inoculated with 2 ml of spore suspension prepared from the culture slants. These flasks were kept in a rotatory shaker (160 rpm) at 35 ^{0}C for 48 hrs. For subsequent studies of submerged fermentation, this induced inoculum was used.

Estimation of gallic acid in the medium using rhodanine

Hagerman (1978) reported that the Gallic acid is assayed by using Rhodanine. The fermented broth is taken in centrifugation tube and centrifuged for 5 min. at 10,000 rpm. 0.025 ml of supernatant liquid is taken into 25 ml, stoppered volumetric flask. 1.5 ml of 0.0667% methanolic Rhodanine solution was added to that, after exactly 5 min. 1ml of 0.5 N aqueous KOH was added.

After 2.5 min. the mixture was diluted with distilled water. 5-10 min. later the absorbance at 520 nm was read against reagent blank. The amount of Gallic acid can be obtained from Gallic acid standard curve.

RESULTS AND DISCUSSION

In the present work, studies on the gallic acid production from myrobalan tannin using three tannase producing fungal strains were carried out. The effect of various parameters at different ranges was studied with different tannase containing strains and their influence on the fermentation was discussed in this chapter. All the experiments were carried out in sequential order with optimized values.

To find out the unknown concentration of Gallic acid produced after Gallo tannin hydrolysis with different tannase rich organisms and also at some important parameters a standard curve is prepared taking gallic acid of analytical grade.

Effect of some important parameters on hydrolysis of gallic acid by using the above three substrates has been studied. Percent hydrolysis of Gallo tannin to gallic acid was calculated on the basis of tannin content of the substrates by the fermentation process.

Effect of fermentation time on Gallic acid production

In order to determine the optimum fermentation time for gallic acid production using three strains, experiments were conducted. The results were tabulated in Table 1 and also Fig. 1. It is evident

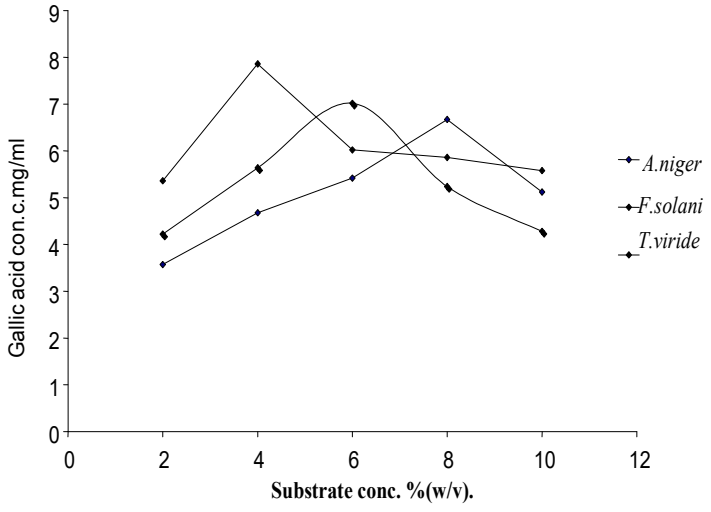

Fig. 1. Effect of substrate concentration on gallic acid production.

Table 1. Comparison of the effect of some parameters on the hydrolysis of gallotannin to Gallic acid by three different fungal strains

Parameters	*Aspergillus niger*	*Fusarium solani*	*Trichoderma viride*
1. Fermentation time (h)			
60	4.02	4.26	7.21
90	6.64	4.36	5.56
120	5.12	5.84	5.22
180	4.56	6.88	4.56
200	4.36	6.32	4.12
2. Substrate Conc %(w/v)			
2 %	3.57	4.22	5.36
4 %	4.91	5.64	7.86
6 %	5.42	7.02	6.02
8 %	6.67	5.24	5.86
10 %	5.12	4.28	5.58
3. pH			
3	4.24	3.98	5.12
4	4.96	4.86	6.62
5	5.06	7.03	7.88
5.5	6.08		
6	5.28	5.52	4.94
4. Temperature (°C)			
25°C	3.88	5.84	5.06
35°C	6.45	7.13	6.24
45°C	4.26	5.24	7.68
55°C	4.08	4.68	4.66
5. Inoculum level (v/v)			
2 ml	3.22	3.58	5.02
4 ml	4.82	4.60	7.98
6 ml	5.84	6.96	5.42
8 ml	6.48	5.36	4.26
10 ml	5.68	5.02	4.26

Table 2. Gallic acid production using all optimized parameters from Myrobalan tannins by *A. niger, F. solani* and *T. viride*

Time in min	Concentration of Gallic acid mg/ml		
	Aspergillus niger	*Fusarium solani*	*Trichoderma viride*
30	3.72	4.26	5.12
60	4.68	5.16	8.28
90	6.88	5.28	606
120	5.02	5.86	4.92
180	4.26	7.28	4.02
200	4.28	5.02	3.63

from Table 1 that increase in the fermentation time from 87 – 175 min increased hydrolysis of Gallo tannin in case of *F. solani,* whereas in *A. niger,* maximum bioconversion was observed at a residence time of 87 min. *T. viride* exhibited peak bioconversion at 60 min fermentation time.

Maximum amount of gallic acid production was observed at 60 min fermentation time in the case of *T. viride* compared to other two strains. Further increase in fermentation time does not increase the gallic acid production; instead there is a slight decrease. This decrease is probably due to the break down of gallic acid by the organism to carbon dioxide and water. Mukherjee, Banerjee (2004) have reported different environmental parameters for more production of tannase and gallic acid using *A. oryzae* and *A. foetidus*. The highest yield of tannase and gallic acid were obtained after 60 hrs by *A. oryzae* and 72 hrs by *A. foetidus.*

Effect of substrate concentration on gallic acid production

The influence of substrate concentration on the production of gallic acid was studied by varying the substrate concentrations viz 2, 4, 6, 8, 10 % (w/v) and the results were tabulated in Table 1 and also shown in Fig. 2.

It can be observed from the results, the optimum substrate concentration was 4% for *T. viride*, 6% for *F. solani* and 8% for *A. niger* and the maximum conversion was obtained. The variation from the reported results may be due to the variation of tannase production in different fungi. It is evident from the Fig. 2. *Trichoderma viride* gave maximum gallic acid when compared to other two strains.

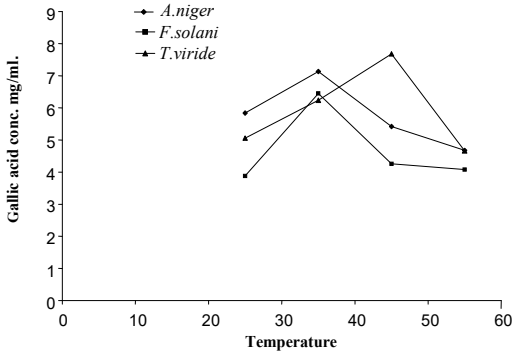

Fig. 2. Effect of temperature on gallic acid production.

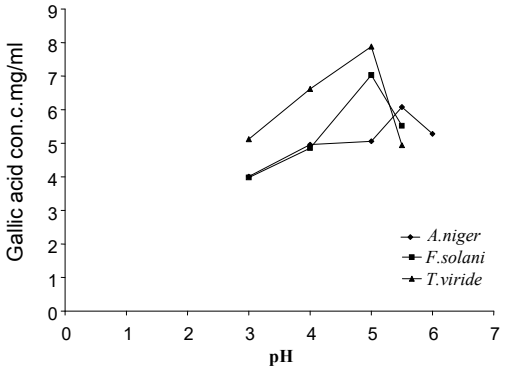

Fig. 3. Effect of pH on gallic acid production.

Chatterjee *et al.* (1996) reported maximum tannase production by *R. oryzae* with an incubation period of 96 hrs under SSF conditions using wheat bran and tannic acid as substrate. Sarma & Singh (2002) reported the isolation of gallic acid from 14 isolates and the ferulic acid in 16 isolates.

Gallic acid production from Tara tannin by *A. niger* has also been reported by Pourrat *et al.* (1985) Optimum production occurred with 15% substrate concentration. The yield of gallic acid was 30% with respect to the weight of raw material.

Effect of pH on gallic acid production

To find out the effect of pH on the gallic acid production, experiments were conducted by varying the pH of the medium from 3 to 6 with an increment of 1 using 0.1N hydrochloric acid and 0.1N sodium hydroxide. The results were tabulated in Table 1 and also shown in Fig. 3. The effect of pH on the production of gallic acid was found to be very important since the pH profile gives a sharp peak. From the Fig. 3 it was understood that *F. solani* and *T. viride* exhibited maximum hydrolysis at initial pH 5 except *A. niger,* where the maximum conversion was obtained at pH 5.5. pH of the reaction medium showed continuous shift towards acidic as the

hydrolysis progressed due to accumulation of gallic acid. Also the source of Gallo tannin is known to alter pH optima of tannase. At pH 5 the *T. viride* gave maximum Gallic production when compared to other two strains.

Bajpai and Patil (1999) reported maximum hydrolysis of gall tannin by *A. fisherii* at pH 6. Optimum pH was reported to be 6.0 by (Pourrat *et al,* 1982) working on a different organism i.e. *A. niger*.

Effect of temperature on Gallic acid production

Experiments were conducted on gallic acid production by varying the temperature from 25°C to 55°C. The results were shown in Table 1 and also shown in Fig. 4. The temperature had a profound effect on gallic acid production.

From the Fig. 4, it can be seen that the optimum temperature for gallic acid production was at 35°C for *A. niger* and *F. solani*. Whereas, *T. viride* gave higher conversion at 45°C. Further increase in the temperature resulted in decreasing gallic acid yields, which could be due to the decreased activity and viability of fungi. This decreased activity and viability may be due to the increase in temperature in the microenvironment.

Optimal temperature was reported to be 55°C by (Naby and Sherif, 1999) working with immobilized enzyme by using *A. oryzae*. Kokai (1982) reported that the gallic acid was produced using *P. chrysogenum. P. chrysogenum* is cultured on a medium containing Tara tannin and the culture broth allowed to stand at 50°C optimum temperature to produce gallic acid.

Effect of inoculum level on Gallic acid production

The effect of inoculum level on Gallic acid production was studied by using different inoculum levels ranging from 2 ml to 10 ml in 50 ml of medium with 2 ml increment using three different strains. The results obtained were given in Table 1 and also shown in Fig. 5.

From the data it is evident that 4 ml is the optimum inoculum level for gallic acid production in the case of *T. viride,* whereas in *F. solani* and *A. niger* the optimum inoculum levels are 6 ml and 8ml respectively. Up to optimum inoculum levels the gallic acid production was increased, later further increase in the inoculum levels resulted in the decrease of gallic acid production. This may be due to the non availability of tannase production. Maximum gallic acid production was obtained from *T. viride*.

The optimum inoculum amount required for maximum tannase and gallic acid production using mineral substrates was 3ml in both cases, i.e., by both *Rhizopus oryzae* and *Aspergillus foetidus.*

Gallic acid production with all optimal conditions using three different fungal strains

The production of Gallic acid from Myrobalan tannins using *T. viride, F. solani* and *A. niger* were carried out. Using the optimal conditions in the

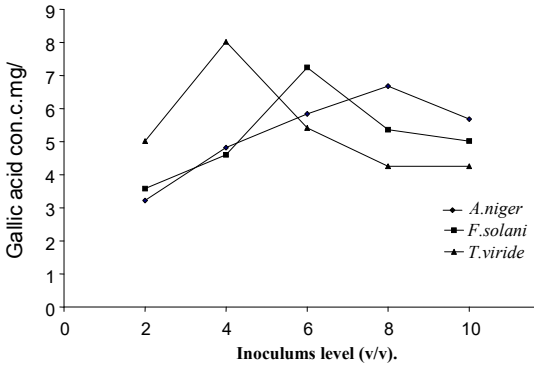

Fig.4. Effect of inoculums level on gallic acid production.

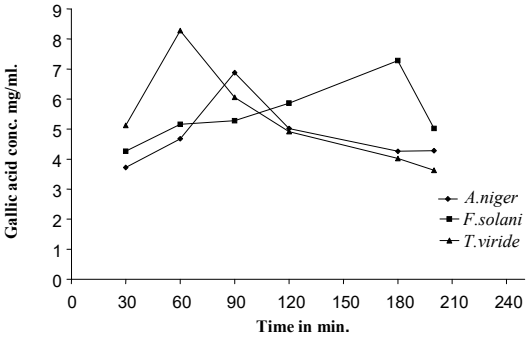

Fig.5. Gallic acid production using all optimized conditions by *Aspergillus niger, Fusarium solani, Trichoderma viride.*

submerged fermentations of these three strains resulted in the higher yield of Gallic acid. This experiment was carried out using all the already estimated optimized values.

The optimal conditions for *T. viride* are substrate concentration 4% (w/v), pH 5.5, temperature 45^{O}C, inoculum level 4 ml, fermentation time 60 min. With the above optimal conditions, *T. viride* exhibited maximum bioconversion of gallic acid, 8.08 mg/ml a time period of after 60 min.

The optimal conditions for *F. solani* are substrate concentration 6 % (w/v), pH 5, temperature 35^{O}C, inoculum level 6 ml, and fermentation time 175 min. With the above optimal conditions, *F. solani* exhibited maximum bioconversion of gallic acid, 7.24 mg/ml a time period of after 175 mins.

The optimal conditions for *A. niger* are substrate concentration 8 % (w/v), pH 5, temperature 35^{O} C, inoculum level 8 ml, fermentation time 87 min. With the above optimal conditions, *A. niger* exhibited maximum bioconversion of gallic acid, 6.88 mg/ml a time period of 87 min.

Gallic acid production at optimal conditions

The experiments were conducted at above all the optimal conditions using these three different fungal strains. The results shown in Table 1 and Fig. 5 indicate that all the three experimental fungal strains exhibited maximum production of gallic acid 87, 175 and 60 min respectively. *T. viride* showed the highest yield of gallic acid 8.08 mg/ml when compared to *A. niger* and *F. solani.*

CONCLUSION

The present work has been taken up with a view to explore the importance of tannase rich fungal strains viz *Trichoderma viride, Fusarium solani* and *Aspergillus niger*, for the production of Gallic acid by using myrobalan tannin. The concentration of Gallic acid produced at all optimized parameters from *Trichoderma viride* is 8.08 mg/ml which corresponds to 78.1% yield, *F. solani* is 7.24 mg/ml which corresponds to 64% yield, and *A. niger* is 6.18 mg/ml which corresponds to 56% yield. Thus this investigation proposes that the *T. viride*, a tannase producing organism can be used for maximum production of Gallic acid.

REFERENCES

1. Abdel M A and Sherif A A,: Gallic acid production from newly isolated organism, *J. App. Micro.,* 1999; **30:** 124-26.
2. Aokik K, Shinke R and Nishira H: Purification and some properties of Yeast tannase. *Agric Biol Chem.,* 1976; **40:** 79 – 85.
3. BajPai B and Patil S: Induction of tannin acyl hydrolase (EC 3.1.1.20) activity in some members of fungi imperfective *Enzyme Microb Technol.,* 1997; **20:** 612-618.
4. Barthomeuf C, Regerat F and Pouurat H: Production and purification of tannase from Aspergillus niger LCF. *J Ferment Bioengg,* 1994; **77:** 320-323.
5. Chae S K and Yu TJ: Experimental manufacture of acorn wine by fungal tannase. *Hanguk Sipkum Kwaha Khoechi,* 1983; **15:** 326-32.
6. Chatterjee R, Dutta A, Banerjee R and Bhattacharaya B C: Production of tannase by solid – state fermentation, *Bioprocess Eng,* 1996; **14:** 159-162.
7. Deschmp A, Otuk G and Lebeault J: Production of tannase and degradation of chestnut tannin by bacteria, *J. Ferment. Technol,* 1983; **61:** 55-59.
8. Haggerman A E and Butler L G: Protein precipitation method for determinations of Tannins, *J. Agric. Food. Chem,* 1978; **26:** 809-812.
9. Hadi T A , Banerjee R and Bhattacharya B C: Optimization of tannase biosynthesis by a newly isolated *Rhizopus oryzae, Bioprocess Engng,* 1994; **11:** 239-243.
10. Haslam E,Haworth R, Jones K and Rogers H: Gallotannins, part 1, The fraction of tannase, *J Chem Soc,* 1961; **9:** 1829-1835.
11. Halsam E and Stangrom J E, The esterase and depsidase activates of tannase, *Biochem*, 1996; **99:** 28-33.
12. Kudson L: Tannic acid fermentation, *J. Biol Chem,* 1913; **14:** 159-202.
13. Lekha P K and Lonsane B K: Production and application of tannin acyl hydrolase, *Adv Appl Microbial,* 1997; **44:** 215 – 260.
14. Mukherjee G and Banerjee R: Production of gallic acid: biotechnological router (part 1), *Chim Oggi Chem Today,* 2003; **21**(1/2): 59 – 62.

15. Nishizawa M, Yamagiashi T, onka G and Nishiokal I: Tannins and related compounds: part 9: isolation and characterization of polygalloyglucoses from Turkish galls (quercer infectoria), *J. Chem. Soc. Perkin – Tranes*, 1983; **5:** 961.
16. Pourrat H, Regrat F, Pourrat A and Jean D, Production of Gallic acid from Tara by a strain of *Aspergillus niger*, *J. Ferment Technol.,* 1985; **63:** 401-405.
17. Rajakumar G S and Nandy S C, Isolation, Purification and some properties of pencillium chrysogenum tannase., *Appl. Environ. Microbial,* 1983; **46**(2): 525 – 527.
18. Van Tieghem: Fermentation gallique Compt. Rend. De'l . *Acad. Des Sci.* 1867; **15:** 1091-4.