Optimization of Process Parameters for the Production of Tannase and Gallic Acid by *Enterobacter Cloacae* MTCC 9125.

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Abstract: Tannase and gallic acid production by *Enterobacter cloacae* MTCC 9125 was optimized. The organism produced maximum enzyme and gallic acid at initial medium pH 4.5 and cultivation temperature of 37^0C after 48 h of incubation period. 1% of 24 h old inoculumn was found to be optimum for tannase production. However, 48 h old inoculumn showed maximum gallic acid accumulation. Supplement of carbohydrates decreased enzyme synthesis, while fructose, sucrose and glucose increases cell mass growth.1.2% tannic acid was found to be optimum for biosynthesis of tannase and gallic acid. The organism showed maximum tannase production with sodium nitrate and KH_2PO_4 as nitrogen source and phosphate source respectively. Ca^{2+} and Mg^{2+} ions were found to be stimulatory for enzyme production. [Journal of American Science. 2010;6(8):389-397]. (ISSN: 1545-1003).

Keywords: *Enterobacter cloacae*, Tannase, Gallic acid, Submerged Fermentation, Enzyme Production

1. Introduction:

Tannase (tannin acyl hydrolase, EC 3.1.1.20) catalyzes the hydrolysis of ester and depside linkages in hydrolyzable tannins like tannic acid. The products of hydrolysis are glucose and gallic acid (Lekha and Lonsane, 1997; Mohapatra et al. 2007). Gallic acid find applications in photography and printing inks, production of an anti-microbial drug trimethoprim, in manufacturing propyl gallate which is used as an antioxidant in fats and oils. Gallic acid also exhibited cytotoxic activity against cancer cells. Besides this, gallic acid possesses wide range of biological activities, such as antibacterial, antiviral, analgesic etc. (Bajpai and Patil 2008; Kar et al. 1999; Mondal et al. 2001; Pourrat et al. 1987; Trevino-Cueto et al. 2007). Other than gallic acid production, tannase is used extensively in the preparation of instant tea, wine, beer, and coffee-flavored soft drinks and also as an additive for detannification of food. A potential use of tannase is in the treatment of waste water contaminated with polyphenolic compounds such as tannic acids (Aguilar et al. 2007; Belmares et al. 2004; Mukherjee and Banerjee 2006; Seth and Chand 2000).

The world wide annual demand of gallic acid is 8000 tons. At present gallic acid is produced industrially by acid hydrolysis of naturally occurring gallotannins. Due to high cost, low yield of desired product and production of large toxic effluent by acid hydrolysis, an enzyme based eco-friendly technology for gallic acid production is urgently required. Microorganisms are known to degrade tannic acid by producing tannases (Bajpai and Patil 2008; Banerjee et al. 2007).

Microorganisms are the main source for industrial enzymes due to their biochemical diversities, ease of cultivation and amenability to genetic modifications (Trevino et al. 2007). Bacteria, yeast and filamentous fungi are known tannase producers. Most of the organisms capable of degrading tannins isolated till date are either anaerobic or facultative anaerobic bacteria from the alimentary canal of ruminating animals or fungal strains associated with the degradation of wood and forest litter. A major problem in the utilization of fungal strains for industrial applications is that degradation by fungi is relatively slow. It is also difficult to manipulate fungal strains genetically because of their complexity. Although several tannin-degrading anaerobic bacteria have been isolated but the processes based on anaerobic bacteria are slow (Chowdhury et al. 2007). Keeping these facts in view, the present communication deals with the production of extracellular tannase and gallic acid by a newly isolated aerobic strain of *Enterobacter cloacae* MTCC 9125.

2.Materials and Methods:

Microorganism: It was isolated from a compost sample and was identified as *Enterobacter cloacae* on the basis of its morphological, physiological and biochemical characteristics (Table 1). The strain has been deposited at MTCC (Microbial Type Culture Collection), IMTECH (Institute of Microbial Technology), Chandigarh, India and has been given accession number 9125.

Table 1. Characterization of *Enterobacter cloacae* MTCC 9125

Colony morphology	
Configuration	Round
Margin	Entire
Elevation	Raised
Surface	Smooth
Pigment	Cream
Opacity	Translucent
Cell Shape	Shorts rods
Size(um)	0.5-1.5u
Arrangement	Occurring singly
Spore(s)	-
Motility	+
Physiological tests	
Growth Temperature	10^0C-42^0C
Growth pH (5-9)	5-9
Growth on NaCl	8%
Anaerobic Growth	(+)
Biochemical tests	
Indole test	-
Methyl red test	-
Voges proskauer test	(+)
Citrate utilization	+
Casein hydrolysis	-
Esculin hydrolysis	(+)
Gelatin hydrolysis	-
Starch hydrolysis	-
Urea hydrolysis	-
Nitrate reduction	+
Catalase test	+
Oxidase test	-
Arginine dihydrolase	+
Tween 20 hydrolysis	+
Tween 40 hydrolysis	+
Tween 80 hydrolysis	(+)
Acid production from	
Glucose	+
Cellobiose	-
Xylose	+
Arabinose	-
Mannitol	+
Maltose	+

+: Positive; -: Negitive; (+): weak positive

Optimization of fermentation process for enzyme production: Various process parameters influencing enzyme production during submerged fermentation were optimized. The strategy followed was to optimize each parameter (cell mass, tannase activity and gallic acid), independent of the others and subsequently optimal conditions were employed in all experiments.

Effect of incubation period and incubation temperature: Fermentation was carried out at various temperatures such as 25, 30, 37, 40 and 45^0C. Samples were withdrawn at regular intervals (12, 24, 36, 48, 60 and 72 h) and analyzed for tannase activity and gallic acid production.

Effect of initial pH: While optimizing the initial pH of the selective medium, the pH of the aqueous solution was varied from 3 to 7 with 1N NaOH or 1N HCl.

Effect of inoculum age and inoculum volume: To determine the effect of inoculum age, the organism was grown at 37 °C for 12, 24, 48, and 72 h in nutrient broth medium (pH 5.0). Then the broth was centrifuged (5,000x*g*, 5 min) and the pellet washed twice in sterilized distilled water and used as inoculum. To determine the effect of inoculum volume, 50 mL of broth was centrifuged and the pellet was dissolved in 1mL of distilled water. A volume of 1, 2.5, 5 and 10 mL of inoculums was used.

Effect of substrate concentration: Various concentrations of tannic acid (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2%) were used as carbon source in the production medium.

Effect of sugar additives: To study the effect of carbon source on enzyme production, simple and complex carbon source like glucose, galactose, mannose, sucrose, lactose and fructose (2.0% w/v) were incorporated in the production medium.

Effect of nitrogen source: Various nitrogen sources such as ammonium chloride, urea, creatinin, sodium nitrate, ammonium nitrate, ammonium molybedate and ammonium thiocynate were added into the medium to determine their effect on cell mass, tannase activity and gallic acid production.

Effect of Phosphate source: The effect of inorganic phosphate (KH_2PO_4) was studied in tannic acid media for tannase and gallic acid production by *E. cloacae* MTCC 9125.

Effect of divalent cations: Different divalent cations (0.01%) were added to study their effect on the enzyme and gallic acid production. These included chlorides of Ca^{2+}, Cu^{2+}, Mg^{2+} and Zn^{2+}.

Tannase assay: Tannase activity was determined colorimetrically using the method of Mondal et al. (2001). The reaction mixture contained 0.3 mL of tannic acid (0.5% in 0.2M sodium acetate buffer, pH 5.5) and 0.1 mL of enzyme, incubated at 50 ^{0}C for 20 min. The enzymatic reaction was stopped by addition of 3 mL of BSA solution, which precipitates the remaining tannic acid. The tubes were centrifuged (5000xg 10 min) and the resultant precipitate was dissolved in 3 mL SDS-triethanolamine solution. One mL of $FeCl_3$ reagent was added to each tube and was kept for 15 min at room temperature for stabilization of the color. The absorbance was read at 530nm using T80 UV/Vis spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme required to hydrolyze 1 mM of tannic acid in 1 min under assay conditions.

Estimation of Gallic acid: Bacterial biomass was obtained by filtration on Whatman filter. The settled solid were then dried overnight at 60 ^{0}C for 24 h and the dry weight expressed in g/L. Gallic acid in the culture filtrate was estimated by the method of Bajpai and Patil (2008). Filtrate was diluted to 100-fold in 0.2 M acetate buffer at pH 5.0. The absorbance was recorded at two selective wavelengths of 254.6 and 293.8 nm. The concentration of gallic acid was measured using specific extinction coefficient by the following equation:

Concentration of gallic acid (µg/mL) =21.77 (A254.6)-17.17 (A293.8).

3. Results and Discussion:

Effect of incubation period and incubation temperature: Tannase and gallic acid production from *E. cloacae* MTCC 9125 was studied in tannic acid medium. Among the different temperatures such as 25, 30, 37, 40 and 45 °C tried, the maximum enzyme and gallic acid production was observed at 37 °C (Table. 2). With further increase in temperature the tannase activity was found to decrease. The organism started enzyme production after 12 h of incubation peaking at 48 h (0.38 U/mL). Optimum temperature of 35-37 ^{0}C was reported for tannase from *L. plantarum* (Ayed and Hamdi 2002) and *A. niger* (Lokeswari and Raju 2007). Maximum gallic acid (3.47 mg/mL) accumulation was also observed within 48 h of incubation period. Sharma et al. (2007), Kar and Banerjee (2000) and Trevino-cueto et al. (2007) found that 48 h of incubation period was optimum for the production of tannase and gallic acid. Rodrigues et al. (2008) reported that tannase is produced during the primary phase of growth. Tannic acid cannot penetrate the cell membrane due to its high molecular weight, but tannase produced by microorganisms can break tannic acid into gallic acid and glucose. The glucose is a readily available carbon source; therefore, it is assimilated first by the microorganism, which results in rapid growth. However, as the glucose concentration falls, gallic acid is utilized by microorganisms as a substrate for energy production.

Table.2. Effect of incubation period and incubation temperature on the production of tannase from *E. cloacae* MTCC 9125.

	Incubation Temperature				
Incubation Time	25°C	30°C	37°C	40°C	45°C
12hr	0.01 (0.41)	0.09 (0.67)	0.10 (0.58)	0.07 (0.56)	0.01 (0.23)
24hr	0.12 (0.64)	0.18 (0.89)	0.21 (1.17)	0.17 (0.85)	0.08 (0.51)
36hr	0.19 (0.82)	0.24 (1.13)	0.31 (2.32)	0.27 (2.01)	0.10 (0.59)
48hr	0.20 (0.84)	0.30 (1.96)	0.38 (3.47)	0.32 (3.18)	0.11 (0.67)
60hr	0.18 (0.77)	0.21 (0.97)	0.25 (1.97)	0.30 (2.68)	0.07 (0.46)
72hr	0.12 (0.69)	0.16 (0.87)	0.18 (1.31)	0.21 (1.17)	0.01 (0.32)

Values in the parenthesis indicating gallic acid production.

Effect of initial pH: The optimum pH for the gallic acid and tannase production was found to be 4.5 (Figure 1). Upon varying the pH of medium from 3.0 to 7.0, the enzyme activity decreased as pH approached the neutrality, whereas highest growth of the organism occurred at pH 5.5. Our results are in accordance with the finding of other reported organisms including fungi (Kar et al. 1999; Chhokar et al. 2009) and bacteria (Mondal and Pati 2000; Mondal et al. 2001).

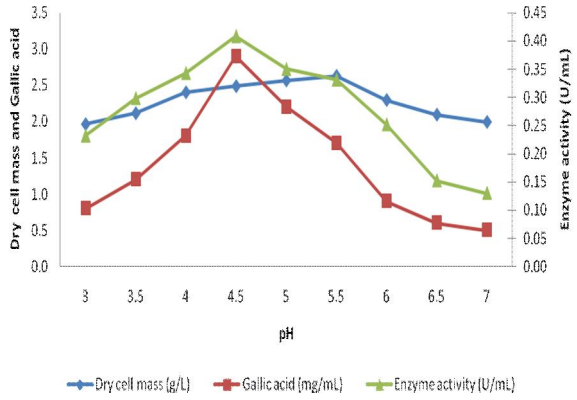

Figure.1 Effect of pH on the production of tannase, gallic acid and growth of E. *cloacae* MTCC 9125.

Naturally any changes in pH may affect the protein structure and a decline in enzyme activity beyond the optimum pH could be due to enzyme inactivation or instability. Tannases have been reported to be acidic proteins, with an optimum pH around 5.5 (Kumar et al. 2007). Effect of pH on the enzyme activity is determined by the nature of the amino acid at the active site, which undergoes protonation and deprotonation and by the conformational changes induced by the ionization of the amino acids (Natarajan and Rajendran 2009).

Effect of inoculum age and size; A 24 h old culture when used as inoculum, gave maximum tannase production in the fermentation medium (Figure 2a.), thereafter the enzyme production declined. However gallic acid and the growth of organism were found to be maximum when 48h old inoculum was used.

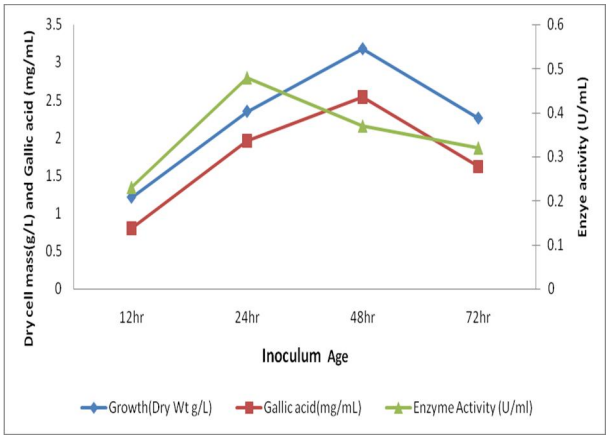

Fig.2a Effect of inoculums age on production of tannase, gallic acid and growth of E. *cloacae* MTCC 9125.

The inoculum level of 1, 2.5, 5 and 10% (v/v) was used in the cultivation medium to establish the effect of inoculum size on the enzyme production by *E. cloacae* MTCC 9125. A 1% (v/v) inoculums (Figure. 2b) was optimal for growth as well as tannase and gallic acid production. Mondal et al. (2001) reported similar results (24 h old culture inoculum) when *Bacillus cereus* KBR9 was used for the production of tannase. Inoculums of 2% (v/v) and 1% have also been reported for the production of tannase by *A. pullulans* DBS66 (Banerjee et al. 2007) and *Lactobacillus sp. ASR- S1* (Sabu et al. 2006) respectively. Lower level of inoculum may not be sufficient for initiating growth and enzyme synthesis on different substrates. An increase in the number of

cells however, ensures a rapid proliferation of biomass and enzyme synthesis. After a certain limit, enzyme production could decrease because of depletion of nutrients due to the enhanced biomass, which would result in a decrease in metabolic activity. A balance between the proliferating bacterial biomass and available substrate material would yield maximum enzyme (Sabu et al. 2006).

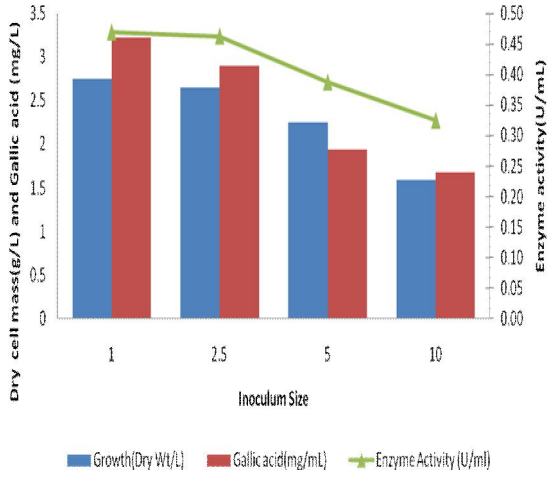

Figure 2b. Effect of inoculums size on production of tannase, gallic acid and growth of E. *cloacae* MTCC 9125.

Effect of substrate concentration: Various concentrations of tannic acid (0.2-1.4%) were used in medium to find out the optimum concentration for tannase and gallic acid production. It was observed that 1.2% tannic acid was suitable for tannase and gallic acid production. (Figure 3). Mohapatra et al. (2009) have reported maximum tannase production by *Bacillus licheniformis* KBR6 in liquid submerged fermentation containing 1% tannic acid. Tannase production from fungal strains were found to be maximum in the medium containing higher concentration of tannic acid (Banerjee et al. 2001; Seth and Chand 2000; Sharma et al. 2007; Paranthaman et al. 2009). However, at higher tannic acid concentration tannase activity was higher in SSF whereas it was repressed in submerged fermentation as tannic acid at higher concentration produces complexes with membrane protein of the organism thereby both growth and enzyme production may be inhibited (Paranthaman et al. 2009). Ayed and Hamdi (2002) reported that increase in substrate concentration induced an increase in tannase activity followed by a decrease because the enzyme synthesis is affected by deposition of gallic acid on the cell surface.

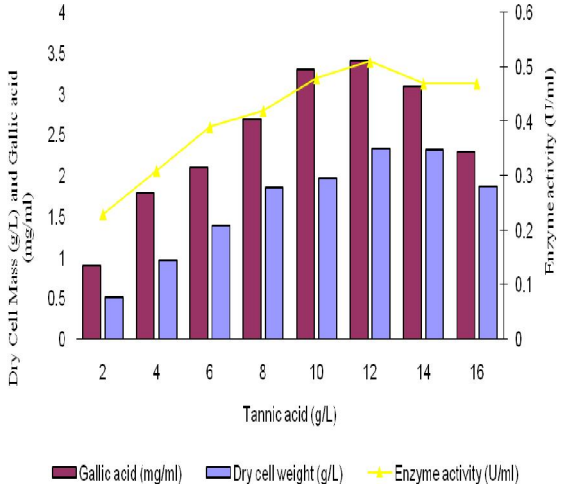

Figure 3. Effect of substrate concentration on the production of tannase, gallic acid and growth of E. *cloacae* MTCC 9125

Effect of sugar additives: Various carbohydrates were added at a concentration of 2 g l^{-1} to the medium containing tannic acid. It was observed that the supplementation of additional carbon sources (glucose, galactose, mannose, sucrose, lactose and fructose) slightly inhibited the enzyme production (Table. 3). Gallic acid synthesis was reduced significantly in the presence of all the additional carbon sources studied.

The presence of glucose decreased the gallic acid production by 54.8% followed by lactose (46.1%), mannose (44.4%), galactose (32.0%), sucrose (25.6%) and fructose (9.8%). However, fructose, glucose and sucrose favored the organism growth. Available reports on the role of carbon sources on the extracellular secretion of tannase are contradictory. Repression of tannase activity by glucose, sucrose, lactose etc. was also reported by Sabu et.al. (2006) and Kumar et al. (2007). Lokeswari and Raju (2007) found that glucose at higher concentration repressed tannase synthesis while the lower concentration is not repressive. This may be due to the fact that high concentrations of additional carbon sources such as glucose changes the carbon/nitrogen ratio as well as creates an osmotic stress, which depresses the enzyme synthesis by microorganisms (Rodrigues et al. 2008). However,

Table.3. Effect of different sugar additives (0.2%, w/v) on growth, gallic acid and tannase production by *E. cloacae* MTCC 9125

Carbo-hydrates	Enzyme Activity (U/mL)	Relative activity (%)	Gallic acid (mg/mL)	Growth (Dry Wt/L)
Control	0.43	100	3.47	1.3
Glucose	0.32	75.0	1.57	1.35
Galactose	0.35	82.0	2.36	1.25
Mannose	0.35	81.0	1.93	1
Sucrose	0.40	92.7	2.58	1.35
Lactose	0.35	80.3	1.87	1.1
Fructose	0.41	94.2	3.13	1.5

Battestin and Macedo (2007) and Bradoo et al. (1997) have reported that external carbon sources did not affect the tannase production. Van de Lagemaat and Pyle (2005) reported that the glucose if present in the media will be exhausted rapidly and this may lead to the partial induction of tannase.

Effect of nitrogen source: Seven different nitrogen sources were used at a concentration of 2 g l^{-1} in a medium containing tannic acid. Among all the nitrogen sources used, sodium nitrate was most effective in tannase biosynthesis and gallic acid production (Table 4) with the relative activity of 171.41% and 266.02% respectively. However, urea favored maximum growth of the organism. Ammonium chloride, urea, creatinin and ammonium nitrate were comparatively less significant for tannase production, presumably because of the release of ammonium ions. Tannic acid by itself could produce only a moderate increase in tannase production whereas by interacting with $NaNO_3$, the tannase activity could be increased significantly (Naidu et al. 2008). Bardoo et. al. (1997) also reported $NaNO_3$ as the preferred nitrogen source for both growth and tannase production by *Asergillus japonicus*. Paranthaman et.al. (2009) observed maximum tannase production by *Aspergillus flavus* in the medium containig $NaNO_3$.

Different concentrations (0.5–5 g l^{-1}) of sodium nitrate were used in the production medium. Optimum concentration of sodium nitrate for the production of tannase and gallic acid was 2 g l^{-1} (Figure 4). Higher concentrations of sodium nitrate in the fermentation medium did not significantly increase enzyme and gallic acid yield. However, maximum growth of the organism was observed at 3 g l^{-1}.

Table 4. Effect of nitrogen sources on the production of tannase and gallic acid from *E. cloacae* MTCC 9125.

Nitrogen Sources	Enzyme Activity (U/mL)	Relative activity (%)	Growth (Dry Wt g/L)	Gallic acid (mg/mL)
Blank	0.22	100	1	1.03
Ammonium Chloride	0.33	148.20	1.41	1.69
Urea	0.32	146.72	2.72	1.57
Creatinin	0.32	145.49	1.19	1.57
Sodium Nitrate	0.38	171.41	1.12	2.74
Ammonium nitrate	0.30	135.79	0.95	0.91
Ammonium Molybedate	0.12	54.90	0.55	0.24
Ammonium Thiocynate	0.21	95.27	1	0.46

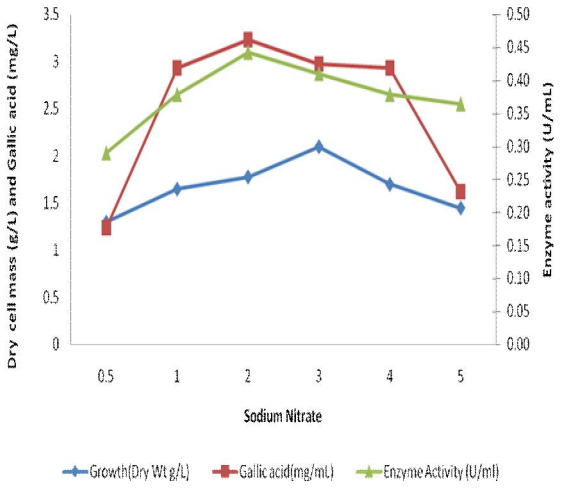

Figure.4 Effect of Sodium Nitrate on production of tannase, gallic acid and growth of E. *cloacae* MTCC 9125.

Effect of metal ions: Cu^{2+}, Zn^{2+}, Ca^{2+} and Mg^{2+} were used in the production medium to determine the effects of metal ions on growth, gallic acid and

tannase production. Cu^{2+} and Zn^{2+} did not affect the enzyme production as well as gallic acid and growth of the organism (data not shown) whereas Ca^{2+} and Mg^{2+} stimulated tannase synthesis. When the concentration of Ca^{2+} and Mg^{2+} (0.05, 0.1, 0.15 and0.2% w/v) was further varied to find out the optimum level it was observed that 0.2% (w/v) Mg^{2+} showed the maximum activity (0.60 U/mL) which accounted for 13.17% and 106.29% increase in enzyme activity and gallic acid production respectively(Table 5). Ca^{2+} at 0.05% (w/v) also supported maximal production of tannase (0.58U/mL). However, gallic acid and growth were maximum at 0.1% (w/v). Whereas at higher concentrations, a small decrease in enzyme activity, growth and gallic acid was observed. This suggests that Mg^{2+} and Ca^{2+} ions are required for the production of tannase by *E. cloacae* MTCC 9125. Natarajan and Rajendran (2009) reported the maximum production of tannase by *Lactobacillus plantarum* MTCC 1407 in the medium containing tannic acid, glucose, NH_4Cl, $MgSO_4$, KH_2PO_4, K_2HPO_4 and $CaCl_2$. Mohapatra et. al. (2009) have also observed the beneficial effect of $MgSO_4$ for tannase production by *Bacillus licheniformis* KBR6.

Effect of Phosphate source:

The results presented in Table 5 indicate that KH_2PO_4 at a concentration of 0.5 g/L enhanced maximum tannase (0.6U/mL) and gallic acid (3.71mg/mL) production with a specific activity of 107.7 and 106.9 respectively. Mohapatra et. al. (2009) observed that phosphate source has strong influence on tannase production by *Bacillus licheniformis* KBR6. Belmares et.al. (2004) reported that the presence of phosphate showed a great importance for optimization, because phosphate promoted the synthesis level and resulted in very expressive decrease in the maximum production time, from 72 to 24 h. The optimized process promoted an increase of 861% in yield and 2783% in productivity. Ayed and Hamdi (2002), Cruz-Hernandez et. al. (2006), Trevino-Cueto et al. (2007), Banerjee et.al. (2007) and Enemuor and Odibo (2009) have also reported beneficial effect of phosphate in the production medium.

Table.5. Effect of Magnesium chloride, calcium chloride and phosphate source on the production of tannase and gallic acid from *E. cloacae* MTCC 9125.

	Calcium Chloride			KH_2PO_4			Magnesium Chloride		
	Enzyme Activity (U/mL)	Gallic acid (mg/mL)	Growth (Dry Wt/L)	Enzyme Activity (U/mL)	Gallic acid (mg/mL)	Growth (Dry Wt/L)	Enzyme Activity (U/mL)	Gallic acid (mg/mL)	Growth (Dry Wt/L)
C	0.55(100.00)	3.02(100.00)	1.78	0.64(100.00)	3.47(100.00)	1.17	0.53(100.00)	1.59(100.00)	1.53
0.5	0.58(111.03)	3.25(107.62)	1.82	0.69(107.71)	3.71(106.92)	1.10	0.57(106.86)	1.68(105.66)	1.78
1	0.54(103.28)	3.30(109.27)	1.86	0.60(94.00)	2.85(82.13)	0.98	0.57(108.46)	1.74(109.43)	1.89
1.5	0.49(93.60)	2.80(92.72)	1.74	0.59(92.81)	2.64(76.02)	1.23	0.59(110.40)	2.87(180.50)	1.97
2	0.34(65.22)	2.20(72.85)	1.63	0.56(87.06)	2.61(75.22)	1.36	0.60(113.17)	3.28(206.29)	1.90

Values in the parenthesis indicating specific activity, C-Control

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