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ARTICLE

Concomitant yield optimization of tannase and gallic acid by *Bacillus licheniformis* KBR6 through submerged fermentation: An industrial approach

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ABSTRACT The present study is concerned with the evaluation of tannase and gallic acid production efficacy of *Bacillus licheniformis* KBR6 under different environmental conditions through submerged fermentation. Results have shown that different environmental conditions and mineral sources have differential influences on tannase and gallic acid production. Highest tannase and gallic acid yield was observed at incubation period of 18 h and 22 h, respectively. At tannic acid concentration of 15 g/l, maximum cell mass (0.75 g/l), cell yield coefficient (0.08 g/g), specific growth rate (37.5 mg/g/h), tannase yield (16.3 U/g) and specific tannase production rate (0.80 U/g/h) were observed, however, at higher tannic acid concentration a decrease in tannase yield and production rate were observed, but gallic acid production increased with increasing tannic acid concentration. Additional carbohydrate sources like glucose, fructose, and lactose showed positive influence on enzyme yield. Among the studied nitrogen sources urea and NH_4Cl, and of the phosphate sources KH_2PO_4 showed favourable effects on cell growth and simultaneous enzyme and gallic acid production. Temperature of 35 °C was found to be optimum for tannase and gallic acid production. Of all the studied metal ions Ca^{2+}, Mg^{2+} and Na^{+} showed positive effect whereas, Co^{2+}, Ag^{2+}, Pb^{2+}, Hg^{2+} showed inhibitory effects.
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Introduction

Tannin acyl hydrolase (E.C. 3.1.1.20), which is commonly called tannase, is an inducible enzyme especially in microbes, produced in the presence of hydrolysable tannins (Barthomeuf et al. 1994; Mondal et al. 2001a; Mukherjee and Banerjee 2006). Tannase hydrolyses the ester and depside linkages of hydrolysable tannins like tannic acid, gallotannins, epigallocatechin-3-gallate, esters of gallic acid into glucose and gallic acid (Iibuchi et al. 1968; Das Mohapatra et al. 2005; Jana et al. 2014).

Tannase has wide applications in food, beverage, brewing, cosmetic and chemical industries (Lekha and Lonsane 1997; Aissam et al. 2005). It is mainly used for the preparation of gallic acid, instant tea, acorn wine, coffee flavoured soft drinks, high-grade leather tannin, clarification of beer and fruit juice, detannification of food and production of wine (Coggon and Sanderson 1972; Lekha and Lonsane 1997; Mukherjee and Banerjee 2003; Aracri et al. 2019; Cavalcanti et al. 2020). It is also used to clean up highly polluting tannin from the effluent of the leather industry (Kim et al. 2020; Biswas et al. 2020).

Besides that, gallic acid (3,4,5, trihydroxy benzoic acid), a hydrolyzable product of tannic acid is an antioxidant and has several uses for the manufacture of propyl gallate, photosensitive resin, pyrogallol, ink and dye (Gaathon et al. 1989; Das Mohapatra et al. 2005; Patil et al. 2011). Conventionally it is produced from tannic acid by acid hydrolysis or by the action of enzyme tannase (Lekha and Lonsane 1997). Microbial process is the most specific, eco-friendly and cost-effective approach and at the same time pure gallic acid could be recovered from the process easily. The world-wide annual demand of gallic acid is about 8000 tones (Lokeswari 2010; Aguilar-Zarate et al. 2015). In India it is totally imported and mainly utilized as an intermediate in the production of trimethoxy benzaldehyde, which is used in the pharmaceutical industry to produce trimethoprim, a broad-spectrum antibiotic (Bajpai and Patil 1997; Aissam et al. 2005; Biswas et al. 2020). A combination of trimethoprim and sulphonamide is effective against many otherwise resistant species of bacteria.

Though tannic acid is generally considered as antinutrient and antimicrobial agent (Scalbert 1991), however, a large number of tannase-producing microorganisms

including bacteria, fungi and yeast can hydrolyse tannic acid into gallic acid and glucose (Mondal and Pati 2000; Banerjee et al. 2001; Sharma et al. 2007; Das Mohapatra et al. 2009). Production of tannase and gallic acid in tannin rich media through fungi has been reported earlier by many researchers (Rajakumar and Nandy 1983; Pourrat et al. 1987; Hadi et al.1994; Bajpai and Patil 1999; Banerjee et al. 2001; Mukherjee and Banerjee 2006). In this context there are comparatively few reports on simultaneous production of the two by bacteria. Using chestnut tannin as carbon source, Deschamps et al. (1983) first reported the production of extracellular tannase by *Bacillus pumilus*, *Bacillus polymixa*, *Corynebacterium* sp. and *Klebsiella pneumoniae*. Later, Deschamps and Lebeault (1984) reported the production of gallic acid from tara pod tannins by *K. pneumoniae* and *Corynebacterium* sp. Tannase-producing strains like *Streptococcus* sp. from faeces of koalas (Osawa and Mitsuoka 1990) and *Lactobacilli* from humans and fermented foods (Osawa et al. 2000) were also reported. Das Mohapatra et al. (2006) reported on tannase production by *Bacillus licheniformis* KBR6 by using eight different (*Acacia auriculiformis, Casuarina quisetifolia, Psidium guazava, Anacardium occidentale, Delonix regia, Eucalyptus tereticornis, Cassia fistula,* and *Ficus bengalensis*) plant extracts as tannin source. Aguilar-Zarate et al. (2015) reported on gallic acid production under anaerobic submerged fermentation by two bacilli strains identified as *Bacillus subtilis* AM1 and *Lactobacillus plantarum* CIR1. A preliminary study was done on isolation of tannase producer from soil, like *B. licheniformis* KBR6 and *Bacillus cereus* KBR9 (Mondal and Pati 2000; Mondal et al. 2001a).

Submerged fermentation involves the growth of the microorganism in a liquid medium in which various nutrients are either dissolved or suspended as particulate solids (Frost and Moss 1987). Submerged fermentation is mostly preferred, because sterilization and process control mechanisms are quite easier (Lekha and Lonsane 1997). In submerged fermentation, uniform growth of the microorganism occurred as a suspension owing to various gases and nutrients, which are either dissolved or suspended in a liquid medium (Frost and Moss 1987).

In this present work the tannase and gallic acid yield efficiency of *B. licheniformis* KBR6 through submerged fermentation has been studied.

Materials and Methods

Microorganism and mode of cultivation

A potent tannase-producing bacterium used in the present study was isolated from the lateritic sal forest soil of Midnapore district, West Bengal, India and was identified as *B. licheniformis* KBR6 (IMI. No.-379224) (Mondal and Pati 2000).

The selective medium used for growth of the organism was composed of (g/l): tannic acid, 10; K_2HPO_4, 0.5; KH_2PO_4, 0.5; $MgSO_4$, 0.5; $CaCl_2$, 1.0; NH_4Cl, 3.0. The pH of sterilized medium was adjusted to 5.0 using 0.5 M NaOH. Cultivation of *B. licheniformis* KBR6 was done in 250 ml Erlenmeyer flask containing 50 ml sterilized medium for 20 h on a rotary shaker (200 rpm) at 35 °C. The culture broth was centrifuged (5000 g for 15 min) and the supernatant was examined for production of tannase as well as gallic acid.

Determination of microbial growth

For measuring the growth, the cell concentration was determined by turbidimetry at 620 nm (SL 171 Mini Spec, Elico, India) and correlated to cell dry weight (mg/ml).

Measurement of tannin concentration

The remaining tannin content of the fermented broth was estimated by the modified method of Hagerman and Butler (1978). The tannin content of the fermented broth (0.5 ml) was precipitated by addition of 3 ml of BSA solution (1 mg/ml) and kept at room temperature for 15 min. After centrifugation (5000 g, 5 min), the precipitate was dissolved in 3 ml of SDS - triethanolamine solution (SDS 1%, w/v, and triethanolamine 5%, v/v, in distilled water). Then 1 ml of $FeCl_3$ reagent (0.01 M $FeCl_3$ in 0.01 N HCl) was added and incubated for 30 min for stabilization of colour. This coloured solution was diluted with distilled water and the absorbency was measured at 530 nm. The residual tannic acid in the fermented broth was determined from a standard curve and expressed as percentage of initial concentration.

Assay of tannase

The activity of extracellular tannase from *B. licheniformis* KBR6 was determined by the colorimetric method of Mondal et al. (2001b). For the assay, 0.1 ml of enzyme was mixed with 0.3 ml of tannic acid substrate solution (1.0% w/v tannic acid in 0.2 M citrate buffer, pH 5.0), and incubated at 50 °C for 30 min. The reaction was terminated by the addition of BSA solution (1 mg/ml), which also precipitated the residual tannic acid. A control reaction with heat-denatured enzyme was performed concomitantly. The tubes were then centrifuged (5000 g, 10 min) and the precipitates were dissolved in 2 ml of SDS-triethanolamine (1% w/v, SDS in 5% v/v, triethanolamine) solution. The absorbance was measured at 530 nm after addition of 1 ml of 0.13 M $FeCl_3$.

The specific extinction coefficient of tannic acid at 530 nm was 0.577 (Mondal et al. 2001b). Using this coefficient, one unit of tannase activity was defined as the amount of enzyme that can hydrolyse 1 μM of ester linkage of

tannic acid in 1 min at 50 ℃ and pH 5.0.

Estimation of gallic acid

The gallic acid in the culture medium was estimated following the method of Bajpai and Patil (1996). Culture supernatant was diluted 100-fold with 0.5 M acetate buffer (pH 6.0) and the absorbance was simultaneously measured at two specific wavelengths. Concentration of gallic acid was calculated by the following equation:

$$\text{Gallic acid } (\mu g/ml) = 21.77\ (A_{254.6}) - 17.17\ (A_{293.8})$$

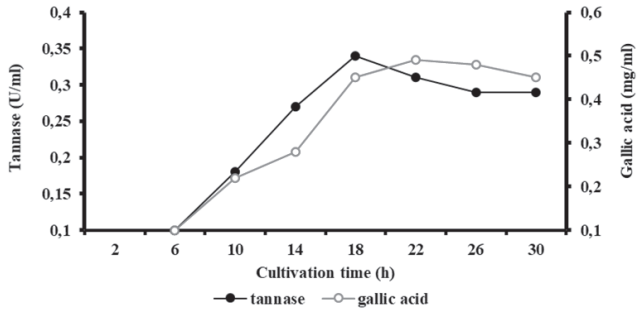

Figure 1. Effect of incubation period on tannase and gallic acid production.

Results

Effect of incubation period

Tannase and gallic acid production in relation to growth of *B. licheniformis* KBR6 were studied in submerged fermentation for 48 h. The formation of tannase was started from the early stages of growth of the bacterium and reached maximum at 18 h. The highest gallic acid production was found at 22 h (Fig. 1).

Effect of tannic acid concentration

The catalytic activity of *B. licheniformis* KBR6 was studied in a fermentation medium containing various tannic acid (sole substrate) concentrations (5 to 40 g/l) and represented in Table 1. Increasing the initial tannic acid concentration from 10 to 15 g/l favoured the growth of the organism. At 15 g/l tannic acid concentration maximum cell mass (0.75 g/l), cell yield co-efficient (0.08 g/g) and specific growth rate (37.5 mg/g/h) of the organism was observed. The highest tannase yield (16.3 U/g) and specific tannase production rate (0.80 U/g/h) were also obtained in this tannic acid concentration. It was also shown that somewhat higher concentration of tannic acid (20 g/l) decreased tannase yield (15.2 U/g) and specific tannase production rate (0.76 U/g/h). But the gallic acid yield coefficient and specific gallic acid production rate increased with increase in tannic acid concentration.

Effect of additional carbohydrate (extra carbon source) in tannic acid medium

Carbon source is known to influence the growth as well as constitutive element in the synthesis of any metabolite. In order to assess the effects of additional carbohydrates on the growth, extracellular tannase formation and gallic acid production by *B. licheniformis* KBR6, four different carbohydrates (2 g/l) viz, glucose, fructose, sucrose and lactose were added to the medium separately and then examined (Table 2). Maximum cell growth, gallic acid yield coefficient and specific growth rate of the organism were obtained in presence of glucose and fructose, whereas tannase yield coefficient and specific tannase production rate showed highest in presence of lactose. In absence of any additional carbon source, the catalytic activity of organism was significantly reduced.

Table 1. Effect of tannic acid concentration on gallic acid, tannase, and cell mass formation by *B. licheniformis* KBR6.

Parameters	Without tannic acid	Tannic acid (g/l)							
		5	10	15	20	25	30	35	40
C_{cm}	0.23	0.66	0.74	0.75	0.68	0.50	0.30	0.30	0.19
$Y_{c/s}$	0.23	0.07	0.07	0.08	0.07	0.05	0.03	0.03	0.02
$Y_{e/s}$	ND	8.1	14.1	16.3	15.2	12.4	11.3	10.9	7.8
$Y_{g/s}$	ND	61.4	75.8	77.5	80.0	80.0	82.0	84.0	83.0
q_e	ND	0.41	0.71	0.80	0.76	0.62	0.57	0.57	0.39
q_g	ND	3.0	3.1	3.8	4.1	4.06	4.1	4.2	4.3
q_c	11	33	37	37.5	34	25	15	15	9
S/s	ND	82.2	83.1	78.0	66.3	46.0	45.8	45.6	32.3

ND = Non detectable; C_{cm} = dry cell mass (g/l); $Y_{c/s}$ = cell yield coefficient (g dry cell mass per g tannic acid used); $Y_{e/s}$ = tannase yield coefficient (U of enzyme per g tannic acid used); $Y_{g/s}$ = gallic acid yield coefficient (g of gallic acid per g tannic acid used); q_e = tannase production rate (U/g/h); q_g = gallic acid production rate (mg/g/h); q_c = growth rate (mg/g/h); **S/s** = tannic acid consumed percentage

Table 2. Effect of carbon source (2 g/l) on tannase, gallic acid and cell mass formation by *B. licheniformis* KBR6.

Parameters	No carbohydrate	Glucose	Fructose	Sucrose	Lactose
C_{cm}	0.41	0.7	0.71	0.67	0.66
$Y_{c/s}$	0.04	0.07	0.07	0.07	0.07
$Y_{e/s}$	3.9	4.5	4.2	6.1	6.5
$Y_{g/s}$	0.125	0.269	0.252	0.216	0.185
q_e	0.195	0.225	0.21	0.305	0.325
q_g	4.1	4.3	4.4	4.4	4.0
q_c	20	35	35	33	33
S/s	66.2	32.2	35.0	40.8	44.2

C_{cm} = dry cell mass (g/l); **$Y_{c/s}$** = cell yield coefficient (g dry cell mass per g tannic acid used); **$Y_{e/s}$** = tannase yield coefficient (U of enzyme per g tannic acid used); **$Y_{g/s}$** = gallic acid yield coefficient (g of gallic acid per g tannic acid used); **q_e** = tannase production rate (U/g/h); **q_g** = gallic acid production rate (mg/g/h); **q_c** = growth rate (mg/g/h); **S/s** = tannic acid consumed percentage

Table 3 Effect of nitrogen source (3 g/l) on tannase, gallic acid and cell mass formation by *B. licheniformis* KBR6.

Parameter	No N_2 source	Urea	NH_4Cl	$(NH_4)_2SO4$	NH_4NO_3	$NaNO_3$	Creatinine	Ammonium oxalate
C_{cm}	0.34	0.78	0.74	0.71	0.73	0.07	0.08	0.07
$Y_{c/s}$	0.03	0.08	0.07	0.07	0.07	0.01	0.01	0.01
$Y_{e/s}$	2.1	9.4	14.8	7.1	11.7	2.8	2.8	5.5
$Y_{g/s}$	0.011	0.052	0.082	0.068	0.08	0.028	0.022	0.022
q_e	0.11	0.47	0.74	0.355	0.585	0.14	0.14	0.275
q_g	ND	2.6	4.1	3.4	4.0	1.4	1.1	1.1
q_c	10	39	37	35	36	3	4	3
S/s	12	63.2	54	43.4	50.1	11.4	12.9	10.9

ND = Non detectable; **C_{cm}** = dry cell mass (g/l); **$Y_{c/s}$** = cell yield coefficient (g dry cell mass per g tannic acid used); **$Y_{e/s}$** = tannase yield coefficient (U of enzyme per g tannic acid used); **$Y_{g/s}$** = gallic acid yield coefficient (g of gallic acid per g tannic acid used); **q_e** = tannase production rate (U/g/h); **q_g** = gallic acid production rate (mg/g/h); **q_c** = growth rate (mg/g/h); **S/s** = tannic acid consumed percentage

Effect of nitrogen source

The nitrogen source is an essential prerequisite for metabolic reaction of any living cell. In order to find out the suitable nitrogen source for tannase and gallic acid production, *B. licheniformis* KBR6 was grown in presence of various nitrogen sources (Table 3), and each nitrogen source was added to the basal medium at a concentration of 3 g/l Urea and NH_4Cl showed a favourable effect in respect to growth of the organism as well as gallic acid and enzyme production. Though the maximum growth of the bacterium occurred in the presence of urea, but the higher enzyme induction was observed in the presence of NH_4Cl. It is also observed that the gallic acid yield coefficient, the specific tannase and gallic acid production rates were also higher in presence of NH_4Cl. In relation to higher growth of bacteria, the tannic acid consumption percentage in presence of urea is much more (63.2%) as compared to other nitrogen sources. A negligible effect was observed in presence of the other nitrogen sources like $NaNO_3$, creatinine and ammonium oxalate.

Effect of phosphate source

The effect of different inorganic phosphates (KH_2PO_4,

Table 4 Effect of phosphate source (0.5 g/l) on tannase, gallic acid and cell mass formation by *B. licheniformis* KBR6.

Parameters	No phosphate source	KH_2PO_4	K_2HPO_4	$(NH_4)_2HPO_4$
C_{cm}	0.12	0.95	0.84	0.62
$Y_{c/s}$	0.01	0.10	0.08	0.06
$Y_{e/s}$	1.0	16.5	14.2	6
$Y_{g/s}$	0.11	0.071	0.069	0.058
q_e	0.015	0.825	0.71	0.3
q_g	1	3.5	3.4	2.9
q_c	6	47	42	31
S/s	1.73	82.2	75.8	45

ND = Non detectable; **C_{cm}** = dry cell mass (g/l); **$Y_{c/s}$** = cell yield coefficient (g dry cell mass per g tannic acid used); **$Y_{e/s}$** = tannase yield coefficient (U of enzyme per g tannic acid used); **$Y_{g/s}$** = gallic acid yield coefficient (g of gallic acid per g tannic acid used); **q_e** = tannase production rate (U/g/h); **q_g** = gallic acid production rate (mg/g/h); **q_c** = growth rate (mg/g/h); **S/s** = tannic acid consumed percentage

Table 5 Effect of temperature on tannase and gallic acid production by *B. licheniformis* KBR6.

Parameter	20 °C	25 °C	30 °C	35 °C	40 °C	45 °C
C_{cm}	0.15	0.25	0.32	0.52	0.27	0.25
$Y_{c/s}$	0.05	0.06	0.07	0.08	0.07	0.06
$Y_{e/s}$	2.9	6.5	11.3	14.4	10.2	5.3
$Y_{g/s}$	0.0	3.3	5.2	6.5	4.5	2.0

C_{cm} = dry cell mass (g/l); $Y_{c/s}$ = cell yield coefficient (g dry cell mass per g tannic acid used); $Y_{e/s}$ = tannase yield coefficient (U of enzyme per g tannic acid used); $Y_{g/s}$ = gallic acid yield coefficient (g of gallic acid per g tannic acid used)

K_2HPO_4 and $(NH_4)_2HPO_4$) was studied for tannase and gallic acid production by *B. licheniformis* KBR6. It has been observed that phosphate source in tannic acid medium is essential for bacterial growth and tannase production (Table 4). In this experiment 0.5 g/l of all the phosphate sources were used. The maximum gallic acid production, tannase yield co-efficient and specific rate of enzyme synthesis by the organism occurred in presence of KH_2PO_4 (Table 4). Whereas, the efficiency of the organism was similar for growth, tannase and gallic acid production in presence of both KH_2PO_4 and K_2HPO_4.

Effect of temperature

To find out optimum temperature for growth and tannase production the organism was grown at different temperature (20-50 °C). It was found that the organism could able to grow in between 20-45 °C. But maximum cell mass growth, tannase and gallic acid yield coefficient were observed at 35 °C (Table 5).

Effect of initial medium pH

Significant growth of organism and tannase and gallic acid production by the organism were observed in the wide range of pH (3.0 to 6.5), but maximum cell mass growth, tannase yield coefficient were found at pH 5.0, whereas highest gallic acid yield coefficient was observed at pH 5.5 (Table 6).

Effect of metal ions

Different cations (0.05%, w/v) were added in tannic acid medium to study their effects on growth, tannase and gallic acid production (Table 7). It has been observed that Ca^{2+}, Mg^{2+} and Na^{+} ions in tannic acid medium increased growth of microorganism, tannase and gallic acid production. Other metal ions like Mn^{2+}, Co^{2+}, Ag^{2+}, Pb^{2+}, Hg^{2+} were inhibitory to bacterial growth as well as tannase and gallic acid production.

Discussion

Simultaneous production of tannase and gallic acid by bacteria has comparatively little report. In comparison to fungal strains, bacteria are highly sensitive to tannic acid (Scalbert 1991). The organism *B. licheniformis* KBR6 produced maximum tannase and gallic acid at its active log phases (18 h and 22 h, respectively). It has also been observed that tannase production was directly proportional to the early (up to 20 h) growth of the organism. In this regard, Deschamp et al. (1983) reported that *Corynebacterium* sp. able to produce maximum tannase at early stages (6 h) of growth but the organism attained highest growth after 24 h. Similar findings were reported by Selwal et al. (2010) where enzyme production by *Pseudomonas aeruginosa* IIIB 8914 was started from its early growth but reached the highest level at 24 h, after which it get decreased.

Increasing the initial tannic acid concentration from 10 to 15 g/l favoured the growth of the organism. Furthermore, increasing substrate concentration (above 15

Table 6 Effect of pH on tannase and gallic acid production by *B. licheniformis* KBR6.

Parameter	3.0	4.0	4.5	5.0	5.5	6.0	6.5
C_{cm}	0.12	0.25	0.32	0.43	0.38	0.28	0.20
$Y_{c/s}$	0.04	0.05	0.06	0.08	0.07	0.05	0.05
$Y_{e/s}$	4.2	5.6	8.2	15.2	9.3	7.5	5.2
$Y_{g/s}$	2.0	3.3	4.2	5.5	6.5	4.2	2.2

C_{cm} = dry cell mass (g/l); $Y_{c/s}$ = cell yield coefficient (g dry cell mass per g tannic acid used); $Y_{e/s}$ = tannase yield coefficient (U of enzyme per g tannic acid used); $Y_{g/s}$ = gallic acid yield coefficient (g of gallic acid per g tannic acid used)

Table 7 Effect of metal ions on growth, tannase and gallic acid production.

Ions (0.05% w/v)	Dry biomass (mg/ml)	Tannase (U/ml)	Gallic acid (mg/ml)
Control	0.35 ± 0.03	0.30 ± 0.06	0.42 ± 0.08
$AgCl_2$	ND	ND	ND
$BaCl_2$	0.12 ± 0.03	0.08 ± 0.02	0.13 ± 0.01
$CaCl_2$	0.36 ± 0.08	0.33 ± 0.04	0.45 ± 0.01
$CoCl_2$	0.13 ± 0.02	0.08 ± 0.03	0.12 ± 0.03
$CuCl_2$	ND	ND	ND
$HgCl_2$	0.09 ± 0.04	0.06 ± 0.01	ND
$MgCl_2$	0.35 ± 0.05	0.37 ± 0.01	0.48 ± 0.05
$MnCl_2$	ND	ND	ND
NaCl	0.42 ± 0.06	0.33 ± 0.08	0.45 ± 0.14
$PbCl_2$	0.18 ± 0.03	0.14 ± 0.06	0.12 ± 0.01
$CaCl_2$+ $MgCl_2$	0.85 ± 0.10	0.35 ± 0.07	0.45 ± 0.03
$MgCl_2$ + NaCl	0.72 ± 0.09	0.36 ± 0.10	0.45 ± 0.04
$CaCl_2$ + NaCl	0.63 ± 0.07	0.31 ± 0.11	0.38 ± 0.02
$MgCl_2$+ NaCl + $CaCl_2$	0.92 ± 0.11	0.43 ± 0.05	0.55 ± 0.09

ND = Non detectable

g/l) resulted significant inhibition of bacterial growth as well as other growth-related parameters. This reveals that growth of *B. licheniformis* KBR6 is highly regulated by the tannic acid concentration in medium. The retardation of growth at higher tannic acid concentration was due to toxicity of substrate itself as it contained large quantity of phenolic groups, which causes precipitation of macromolecules including proteins and carbohydrates (Lewis and Starkey 1969; Scalbert 1991). In this experiment, an increment in the specific production rate and yield of gallic acid was observed with increasing the initial tannic acid concentration up to 20 g/l and after which there were no significant alteration observed with further enhancement of tannic acid. The initial rapid production of gallic acid was due to increase in growth as well as higher tannase in the medium. Above 20 g/l of tannic acid both growth and tannase synthesis inhibited but due to availability of substrate (tannic acid) to low concentration of enzyme, the gallic acid formation rate was not drastically altered. In the present study, an initial tannic acid concentration of 15 g/l was found to be a better tannase inducer than 10 or 20 g/l. Earlier most of the reports indicated that a specific tannic acid concentration is suitable for microbial tannase biosynthesis (Hadi et al. 1994; Bradoo et al. 1997). Raghuwanshi et al. (2011) reported maximum tannase production (11.2 IU/ml) in medium containing 2.0% tannic acid by *Bacillus sphaericus*.

The formation of extracellular enzyme is found to be strongly affected by the nature of additional carbon source used. The addition of lactose in tannic acid media enhanced tannase production about 1.7-fold. The requirement of additional carbon sources for tannase synthesis by fungal strains in tannic acid media have been reported by many workers (Hadi et al. 1994; Bradoo et al. 1997). Hadi et al. (1994) mentioned that carbohydrates act as a catabolic inducer for tannase biosynthesis in *Rhizopus oryzae*. Mondal et al. (2000) and Mondal and Pati (2000) observed that the addition of low concentrations of additional carbon sources like glucose, lactose and sucrose (0.1%) were supportive for enzyme production by *B. licheniformis* KBR6.

The nitrogen source in the culture medium is very essential for microbial growth. Organisms assimilate specific nitrogen source from their surrounding environment and mainly use it as a precursor of amino acid as well as cellular protein synthesis. In this experiment, the bacterium *B. licheniformis* KBR6 synthesized more tannase as well as gallic acids in presence of NH_4Cl. Lekha and Lonsane (1997) have also reported the nutritional requirement of specific nitrogen source in culture media for fungal tannase production. Sabu et al. (2006) reported an increase in the tannase production by *Lactobacillus* sp. ASR-S1 with NH_4NO_3 supplement in the medium containing tamarind seed powder (TSP). Belur et al. (2010a) observed enhanced tannase production by *Serratia ficaria* in presence of casein hydrolysate and yeast extract with NH_4NO_3 in fermentation medium. Raghuwanshi et al. (2011) observed maximum tannase production by *Bacillus sphaericus* with 0.25% ammonium chloride. Organic nitrogen sources (beef extract, peptone, etc.) were not used in the medium as these form insoluble complexes with tannic acid. Added advantages of inorganic nitrogen

sources are that, they are less expensive than organic one and at the same time they avoid the problems of complex formation during enzyme purification.

The inorganic phosphate sources are very much essential for tannase production from *B. licheniformis* KBR6. Different phosphate compounds in the basal medium for tannase production have been found in earlier reports (Hadi et al. 1994; Bradoo et al. 1997; Lekha and Lonsane 1997). This report is in accordance with the results reported by Das Mohapatra et al. (2009), where enzyme production by *B. licheniformis* KBR6 was optimised using KH_2PO_4.

It has been found that Ca^{2+}, Mg^{2+} and Na^{+} stimulated the tannase production by *B. licheniformis* KBR6. Micro- and macroelements act as an elementary composition of cell, but a particular ion has stimulatory effect to metabolic synthesis in specific group of microorganisms (Schlegel 1995). Beniwal et al. (2010) showed stimulatory effect of Ca^{2+} and Mg^{2+} ions for enzyme production by *Enterobacter cloacae* MTCC 9125.

Tannase is a most promising and applicable microbial enzyme in bioprocess industry. The enzymatic by product, gallic acid has also many uses in chemical industry. Both tannase and gallic acid can be produced by *B. licheniformis* KBR6 in tannic acid containing culture media. The optimal composition of this medium was tannic acid (15 g/l), glucose (2 g/l), NH_4Cl (3 g/l), KH_2PO_4 (0.5 g/l) and $MgCl_2$ (0.5 g/l). In this minimal medium bacterium can produce tannase in large amounts at short period of cultivation in comparison to other tannase producing fungi.

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