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Biosynthesis of tannin acyl hydrolase from tannin-rich forest residue under different fermentation conditions

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Caesalpinia digyna, a tannin-rich forest residue, was used as substrate for production of tannase and gallic acid. Media engineering was carried out under solid-state fermentation, submerged fermentation and modified solid state fermentation conditions for optimum synthesis of tannase and gallic acid (based on 58% tannin content in the raw material). Tannase vis-à-vis gallic acid recovery under modified solid-state fermentation condition was maximum. Conversions of tannin to gallic acid under solid-state fermentation, submerged fermentation and modified solid-state fermentation conditions were 30.5%, 27.5% and 90.9%, respectively. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 29–38.

Keywords: tannase; *Caesalpinia digyna*; *Rhizopus oryzae*; modified solid state fermentation

Introduction

Tannin acyl hydrolase, commonly referred to as tannase (EC 3.1.1.20), is used for the bioconversion of tannin to gallic acid. Tannase is present in many tannin-rich plant materials, such as myrobalan (*Terminalia chebula*) fruits, divi divi (*Caesalpinia coriaria*) pods, dhawa (*Anogeissus latifolia*) leaves and the bark of konnam (*Cassia fistula*), babul (*Acacia arabica*) and avarum (*Cassia auriculata*) trees [14]. Although many enzymes are obtained from animal and plant sources [5], microorganisms are the favoured source for production of industrial enzymes because of their biochemical diversity and their technical and economic advantages [18]. Microorganisms can be cultured in large quantities in a short time by established methods of fermentation. Thus, they can produce an abundant and regular supply of the desired enzyme. Microbial enzymes are more stable than analogous proteins obtained from plant and animal sources. Microbes can also be subjected to genetic manipulation more readily than plants and animals [19].

Tannase is an extracellular, inducible enzyme, that catalyses the hydrolysis of ester and depside bonds only in hydrolysable tannins (does not act on condensed tannins), releasing gallic acid and glucose [4].

Gallic acid (3,4,5-trihydroxy benzoic acid) is a phenolic compound. Its major use is in pharmaceutical industries for manufacture of trimethoprim (TMP), an antibacterial agent, which is usually given along with sulphonamide; together they have a broad spectrum of action. The consumption coefficient of gallic acid in the manufacture of trimethoprim is 4.8 [6]. Gallic acid is also used in enzymatic synthesis of gallic acid esters, e.g., propyl gallate, which is used mainly as an antioxidant in fats and oils, as well as in beverages. Further, it also finds use in the leather industry and in the manufacture of pyrogallol. Pyrogallol is used in staining fur, leather and hair and also as photographic developer [12].

The production of tannase has been studied with different fungal species and it was observed that *Rhizopus oryzae* (RO IITKGP RB-13, NRRL21498), which was isolated from soil on the IIT campus, produces an appreciable amount of tannase [10]. As reports on the synthesis of tannase and production of gallic acid from *Caesalpinia digyna* seed cover powder are very limited, efforts have been made to optimize various physicochemical parameters for the maximum production of tannase and gallic acid under different fermentation conditions.

Materials and methods

Raw material

C. digyna (known locally as gilo) seed cover is found in abundance in the eastern part of India. It was first dried at 60°C in an oven to remove free moisture. The material was then finely ground in a grinder and screened to collect powder of 76 μm particle size. The oversized particles were recycled. The powder was stored in a dry place at room temperature.

Microorganism

The Phycomycete *R. oryzae* was isolated from soil of the IIT campus and was maintained on 2% malt extract agar slants.

Chemicals

All chemicals used were of analytical grade.

Inoculum preparation

Tannase being an inducible enzyme, preinduced inoculum was prepared using modified Czapek–Dox medium ($NaNO_3$ 0.25%, KH_2PO_4 0.1%, $MgSO_4$ 0.05%, KCl 0.05% and *C. digyna* seed cover powder 2.0% as sole carbon source) [3].

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Table 1 Optimum conditions for maximum tannase and gallic acid production

Serial No.	Parameters	SSF		SmF		MSSF	
		Range	Optimum	Range	Optimum	Range	Optimum
1	Incubation period (h)	24–96	72	24–96	48	24–96	72
2	Substrate content	0.5–4/63.6 (g/cm^2)	2.0	2.5–20 (% (w/v))	10	5–40/72 (g/cm^2)	20
3	Initial pH	3–6.5	4.5	3–6.5	5.0	3–6.5	4.5
4	Temperature (°C)	20–40	32	20–40	37	20–40	32
5	Relative humidity (%)	67–94	93			67–94	93
6	Bed height (cm)	0.01–0.2	0.15			0.05–2.3	1.5
7	Solid:liquid ratio	0.5:1–4:1	1:1	0.05:1–1:1	0.4:1	0.067:1–0.8:1	0.4:1
8	Condition of fermentation		Stationary	Stationary/Agitation	Agitation	Stationary/Agitation	Stationary
9	Selection of media (a) Czapek–Dox (with 2% (w/v) glucose) (b) Modified Czapek–Dox (with 2% (w/v) tannic acid) (c) Modified Czapek–Dox (with 2% (w/v) *C. digyna* powder) (d) Tannic acid medium		(c)		(c)		(c)
10	'Tannase activity (U/ml)		18.87		23.86		32.76
11	Yield of gallic acid based on tannin content in *C. digyna* (%)		30.5		27.5		90.9

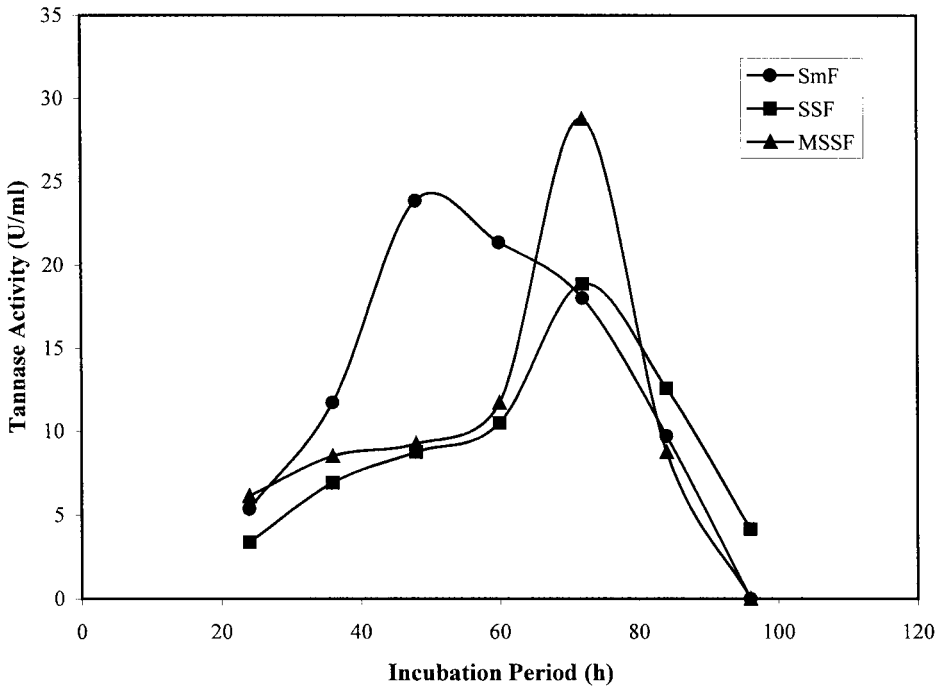

Figure 1 Effect of incubation period on tannase production under SSF, SmF and MSSF conditions at constant temperature (32°C SSF and MSSF, 37°C SmF), pH (4.5 SSF and MSSF, 5.0 SmF), substrate content (2 g SSF, 10% (w/v) SmF, 20 g MSSF), relative humidity (93% SSF and MSSF), solid:liquid ratio (1:1 SSF, 0.4:1 SmF and MSSF), bed height (0.15 cm SSF, 1.5 cm MSSF).

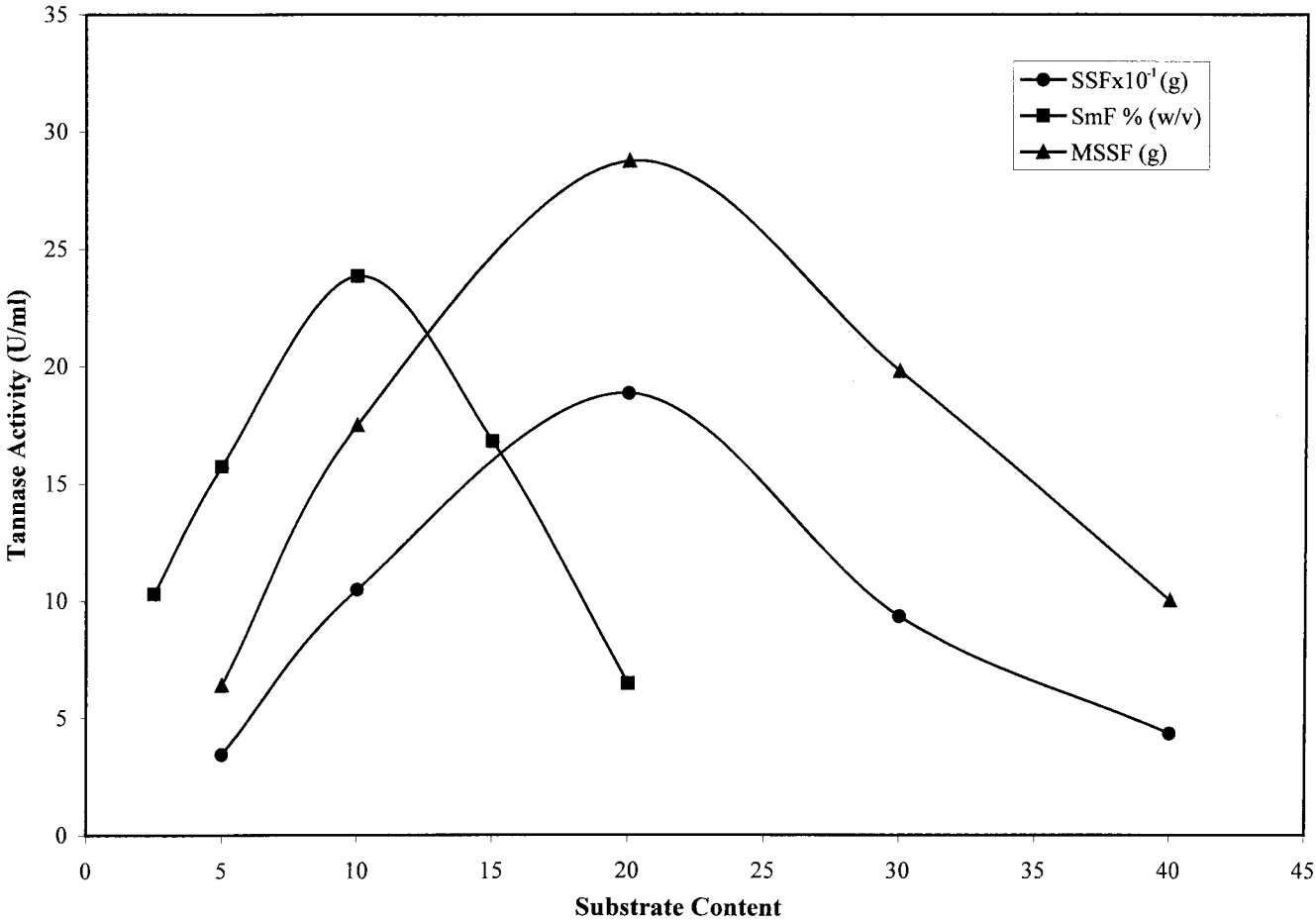

Figure 2 Effect of substrate content on tannase production under SSF, SmF and MSSF conditions at a constant incubation period (48 h SmF, 72 h SSF and MSSF), temperature (32°C SSF and MSSF, 37°C SmF), pH (4.5 SSF and MSSF, 5.0 SmF), relative humidity (93% SSF and MSSF), solid:liquid ratio (1:1 SSF, 0.4:1 SmF and MSSF), bed height (0.15 cm SSF, 1.5 cm MSSF).

Tannin estimation

Total tannin content of the raw material was estimated following the procedure given by Hagerman and Butler [8] with bovine serum albumin (BSA) as protein standard. *C. digyna* seed cover powder contains 58% tannin.

Tannase production

Solid state fermentation (SSF): Powdered *C. digyna* seed cover (2 g) in a petri plate to which an equal volume of modified Czapek–Dox medium was added and mixed thoroughly with a spatula. It was autoclaved at 121°C for 15 min and after cooling it was inoculated with 1 ml of a preinduced culture of *R. oryzae* and incubated at 32°C and 93% relative humidity for enzyme production. After 72 h, the fermented solid from the petri plate was transferred to a 250-ml beaker, mixed thoroughly with deionized water (2×volume, based on weight of the substrate after fermentation) and kept for 2 h at room temperature. The contents of the petri plate were squeezed through cheesecloth. After extracting, the extract was centrifuged for 10 min at 10,000×*g* and the clear supernatant was taken as crude enzyme for assay.

Submerged fermentation (SmF): Powdered *C. digyna* seed cover (10%, w/v) was taken in a 250-conical flask, containing 100 ml of modified Czapek–Dox medium. It was autoclaved at 121°C for 15 min and after cooling it was inoculated with 1 ml of induced inoculum and incubated at 37°C with shaking. After 48 h, the culture broth was centrifuged at 10,000×*g* for 10 min to separate the mycelial mass and the supernatant containing the enzyme was taken for assay.

Modified solid state fermentation (MSSF): Powdered *C. digyna* seed cover (20 g) was placed on the mesh of a GROWTEK bioreactor [11]. Modified Czapek–Dox medium (50 ml) was added beneath the mesh of the bioreactor. It was autoclaved at 121°C for 15 min and after cooling 1 ml of induced inoculum was added to the substrate (on mesh) and mixed thoroughly with a sterile spatula. The bioreactor was then incubated at 32°C and 93% relative humidity for 72 h. After incubation, the fermented biomass and the modified Czapek–Dox medium were transferred to a 500-ml beaker and mixed thoroughly followed by centrifugation at 10,000×*g* for 10 min to remove the mycelial mass. The supernatant was used as crude enzyme for assay.

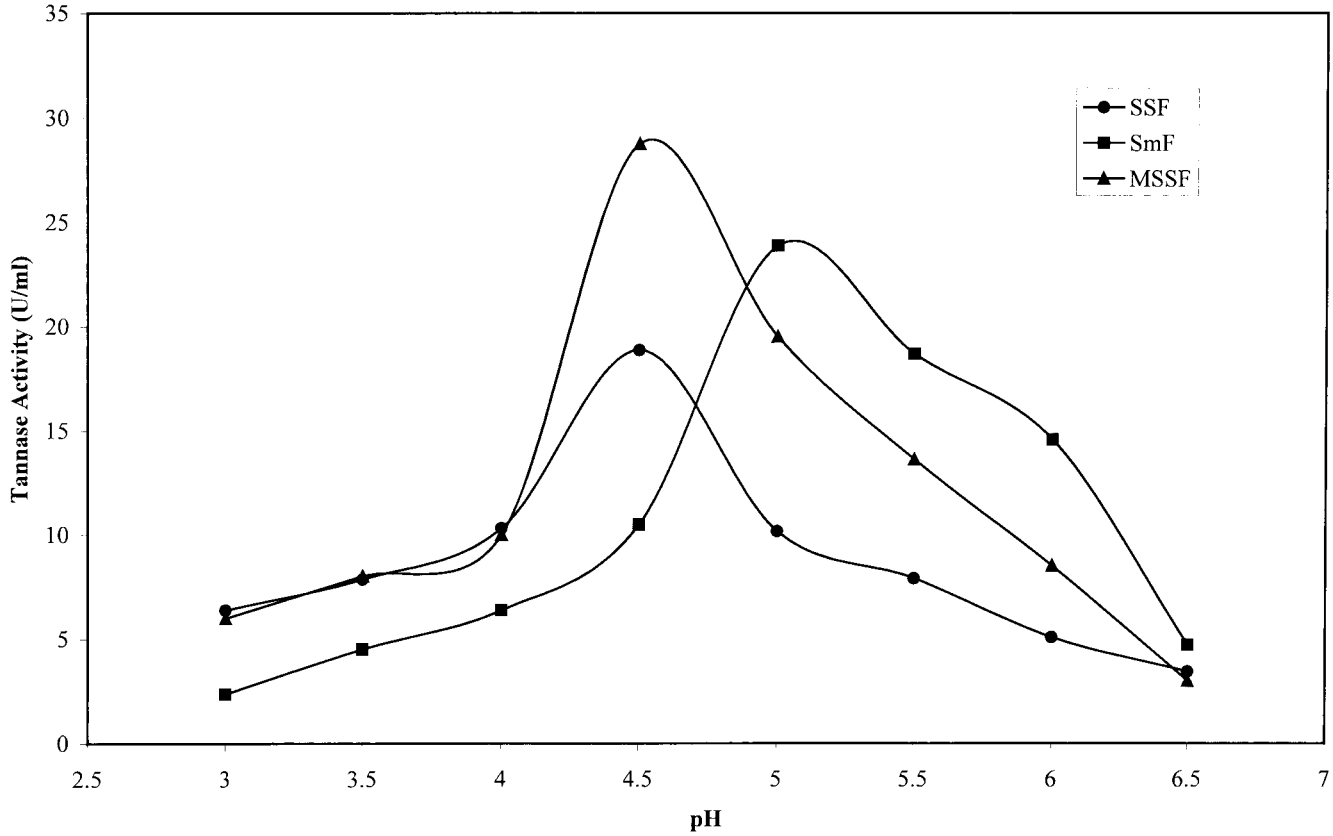

Figure 3 Effect of initial pH on tannase production under SSF, SmF and MSSF conditions at a constant incubation period (48 h SmF, 72 h SSF and MSSF), substrate content (2 g SSF, 10% (w/v) SmF, 20 g MSSF), temperature (32°C SSF and MSSF, 37°C SmF), relative humidity (93% SSF and MSSF), solid:liquid ratio (1:1 SSF, 0.4:1 SmF and MSSF), bed height (0.15 cm SSF, 1.5 cm MSSF).

Tannase assay

Tannase activity was determined spectrophotometrically [9]. One unit of enzyme activity is defined as the amount of enzyme required to hydrolyse 1 μmol of ester in 1 min. Enzyme activity values are expressed as units/ml (U/ml).

Gallic acid extraction

Gallic acid extraction was carried out as suggested by Kar *et al.* [11].

Media engineering influencing tannase production

The protocol adopted for the optimization of fermentation parameters was to study the effect of an individual parameter and to incorporate it at the optimized level before optimizing the next parameter [16]. The effect of incubation period was studied in the range of 24–96 h under all three conditions of fermentation. Similarly, the initial pH of the medium was evaluated in the range of 3–6.5. The effect of incubation temperature was observed in the range of 20–40°C. The effect of substrate content per square centimeter was studied in the range of 0.5–4.0 g in the case of SSF, 2.5–20% (w/v) in SmF and 5–40 g in MSSF. The influence of relative humidity was evaluated in the range of 67–94% in SSF and MSSF. The effect of solid:liquid ratio was studied in the range of 0.5:1–4:1 in SSF, 0.05:1–1:1 in SmF and 0.067:1–0.8:1 in MSSF. Finally, the effect of bed height was evaluated in the range of 0.01–0.2 cm in SSF and 0.05–2.3 cm in MSSF. The fermentation was varied between stationary and agitated conditions in SmF and MSSF. All experiments were conducted in triplicate and average values are reported. The optimized values were repeated thrice in order to corroborate their validity (Table 1).

Results

The results obtained under the three fermentation conditions are summarized in Table 1.

The optimum incubation period under SSF and MSSF conditions was 72 h whereas for the SmF process it was only 48 h (Figure 1), which indicates that mixing has a significant influence on tannase synthesis.

Two grams of substrate in SSF, 10% (w/v) in SmF and 20 g in MSSF gave optimum tannase activity (Figure 2).

The optimum pH for the synthesis of tannase was 4.5 under SSF and MSSF conditions and 5.0 under SmF conditions when the variation was from 3.0–6.5 (Figure 3).

A temperature of 32°C under SSF and MSSF conditions and 37°C under SmF conditions was most favourable for tannase production when the temperature was varied from

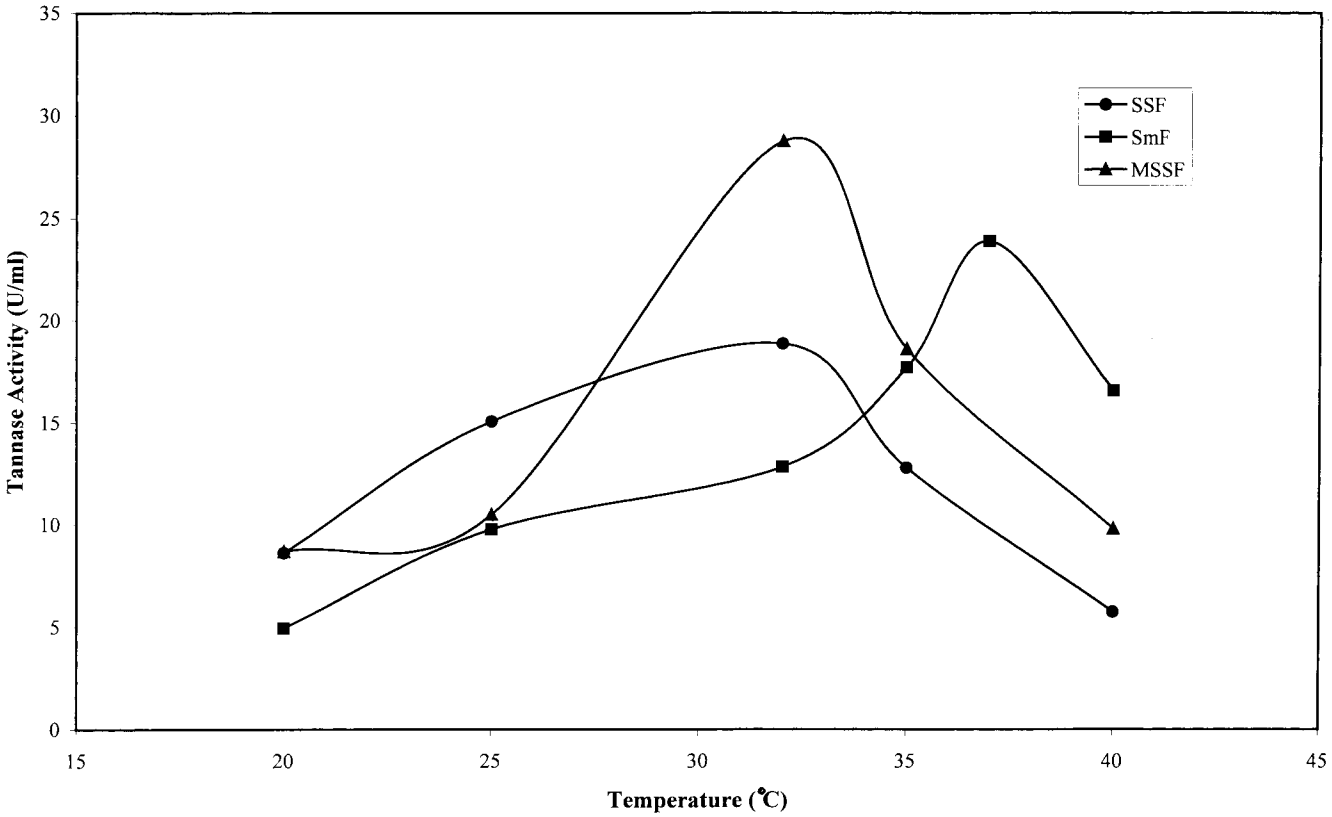

Figure 4 Effect of temperature on tannase production under SSF, SmF and MSSF conditions at a constant incubation period (48 h SmF, 72 h SSF and MSSF), substrate content (2 g SSF, 10% (w/v) SmF, 20 g MSSF), pH (4.5 SSF and MSSF, 5.0 SmF), relative humidity 93% SSF and MSSF), solid:liquid ratio (1:1 SSF, 0.4:1 SmF and MSSF), bed height (0.15 cm SSF, 1.5 cm MSSF).

20–40°C (Figure 4). Tannase activity increased initially up to 32°C under SSF and MSSF conditions and 37°C under SmF conditions and thereafter decreased with a rise in temperature.

There was no significant difference in the tannase synthesis in SSF and MSSF conditions at 67% and 76% relative humidity (Figure 5). Thereafter up to 93% relative humidity a significant increase in tannase synthesis was observed under MSSF conditions

A bed height of 1.5 cm (Figure 6) with a solid:liquid ratio of 0.4:1 showed optimum tannase production under SmF and MSSF conditions whereas under SSF conditions, 1:1 solid:liquid ratio with 0.15 cm bed height supported optimum tannase synthesis.

Under stationary MSSF conditions, an incubation period of 72 h gave optimum tannase production whereas under agitated MSSF conditions, tannase synthesis was maximum with 48 h of incubation but the yield of gallic acid was considerably less (Figure 7). Under SSF conditions, 72 h gave optimum tannase synthesis under stationary fermentation whereas 48 h yielded optimum tannase synthesis under agitated conditions in the SmF process.

Tannase and gallic acid production were better with modified Czapek–Dox medium (containing 2% *C. digyna* seed cover powder) compared to tannic acid medium and other combinations of modified Czapek–Dox medium (Figure 8).

Discussion

Tannase synthesis and gallic acid yield are directly proportional i.e., maximum synthesis of tannase results in maximum yield of gallic acid.

The reason for the initial increase in tannase activity followed by a decrease (Figure 1) is catabolite repression [2] as well as substrate scarcity and secretion of toxic substances, e.g., catechuic acid, 2,6-dihydroxy benzoic acid, and pyrogallol which could cause cell lysis [11] or it may be due to metabolic regulation which is related to the release of gallic acid [4]. Chatterjee *et al.* [2] showed that maximum tannase synthesis was obtained with 4 days of incubation under SSF conditions whereas Hadi *et al.* [7] observed it on the 5th day under SmF conditions using *R. oryzae*. Similarly, Lekha and Lonsane [12] reported maximum tannase synthesis with 4 days and 6 days of incubation under SSF and SmF conditions respectively, when *Aspergillus niger* PKL 104 was used. In the present study, an incubation period of 3 days offered advantages in terms of the overall economy of tannase production.

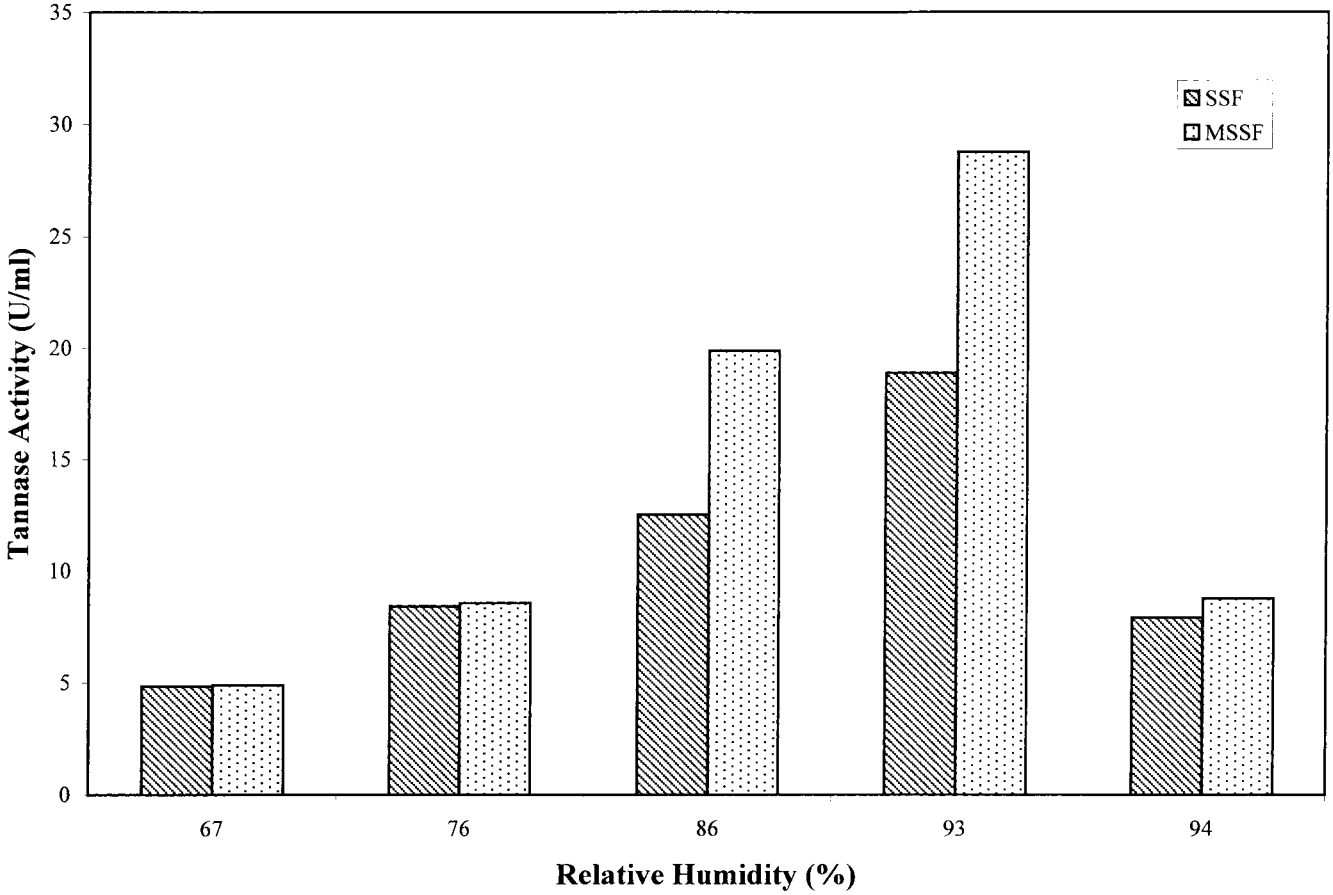

Figure 5 Effect of relative humidity on tannase production under SSF and MSSF conditions at a constant incubation period (48 h SmF, 72 h SSF and MSSF), substrate content (2 g SSF, 10% (w/v) SmF and 20 g MSSF), pH (4.5 SSF and MSSF, 5.0 SmF), temperature (32°C SSF and MSSF, 37°C SmF), solid:liquid ratio (1:1 SSF, 0.4:1 SmF and MSSF), bed height (0.15 cm SSF, 1.5 cm MSSF).

A number of factors could be responsible for the decline in tannase activity with increasing substrate quantity shown in Figure 2. First, the intermediate hydrolysates, namely 1,2,3,4,6-pentagalloyl glucose, 2,3,4,6-tetragalloyl glucose, and monogalloyl glucose produced during hydrolysis of tannic acid could be binding competitively or noncompetitively with the enzyme sites. Gallic acid itself acts as a competitive inhibitor [11]. Second, generation of heat with the increase in substrate quantity is a common phenomenon under SSF and MSSF conditions, which results in the denaturation of some of the heat-sensitive biochemical products produced during fermentation. Growth of the microorganism is also adversely affected by the heat generated under MSSF conditions, an attempt was made to overcome the problem of heat generation by keeping the solid (on float) in continuous contact with the liquid (below the float), which gradually absorbs the excess heat generated.

Under SSF and MSSF conditions, an initial pH of 4.5 gave maximum tannase production (Figure 3) whereas the report by Chatterjee *et al.* [2] and Lekha and Lonsane [12] showed that initial pHs of 5.0 and 5.5 gave maximum enzyme production under SSF condition using *R. oryzae* and *A. niger* PKL 104, respectively. In SmF, an initial pH of 5.0 gave maximum tannase production. The enzymes contain both positively and negatively charged groups at their active sites which are activated only at a particular pH, which in turn influences product formation [1].

Tannase production under SSF conditions was best at 40°C [2] but in the present study maximum enzyme activity was obtained at 32°C when tannin-rich agro residue, *C. digyna*, was the substrate. The reason for the initial rise in tannase activity followed by a decrease thereafter (Figure 4) is that the atoms in the enzyme molecule have greater energy and a greater tendency to move with increased temperature. Therefore, up to a certain temperature they acquire sufficient energy to overcome the weak interactions holding the globular protein structure together and deactivation follows [17].

Relative humidity also plays an important role in enzyme production. In SSF condition, as fermentation proceeds heat is generated and subsequently evaporation takes place, which is taken care of by the excess liquid in the vessel under MSSF conditions. Relative humidity helps to maintain water content within the fermentation media. With increased relative humidity, dryness of the bed is reduced which facilitates entry of nutrients into the cell which favours enzyme production. With further increase in relative humidity, as in the SmF process, enzyme activity decreases which

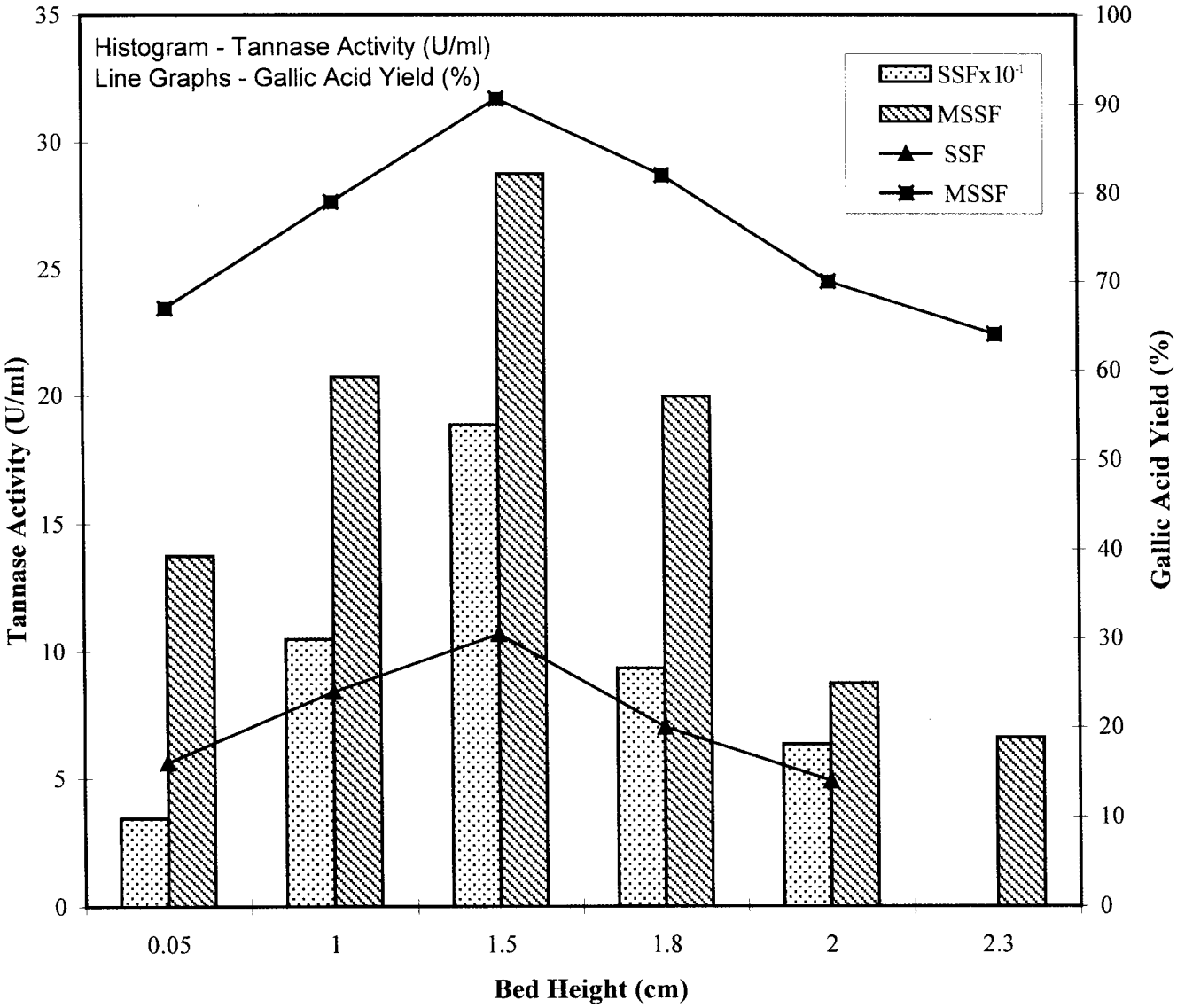

Figure 6 Effect of bed height on tannase production under SSF, SmF and MSSF conditions at a constant incubation period (48 h SmF, 72 h SSF and MSSF), substrate content (2 g SSF, 10% (w/v) SmF, 20 g MSSF), pH (4.5 SSF and MSSF, 5.0 SmF), temperature (32°C SSF and MSSF, 37°C SmF) solid:liquid ratio (1:1 SSF, 0.4:1 SmF and MSSF), relative humidity (93% for SSF and MSSF).

could be due to osmotic imbalance within the cell leading to lysis (Figure 5).

With the increase in substrate quantity under SSF and MSSF conditions, the bed height also increased and subsequently gallic acid production decreased. This could be due to the fact that, with the increase in bed height under MSSF conditions, the liquid in the vessel in contact with the solid substrate containing the enzyme on the float may not be sufficient to convert the entire substrate into gallic acid or aid in leaching of the entire product into the medium. Further, with the increase in bed height (keeping the contact area constant) the compactness of the substrate is increased resulting in lessening of the mass transfer process, which in turn inhibits mycelial penetration, and nonuniform distribution of the extracellular enzyme throughout the bed. Therefore, an optimum bed height of 1.5 cm (Figure 6) with a solid:liquid ratio of 0.4:1 gave maximum yield of gallic acid.

Agitated conditions are unfavourable for tannase as well as gallic acid production (Figure 7). Although, *R. oryzae* is aerobic, it can grow under low oxygen tension. Under agitated MSSF conditions, tannase synthesis is faster than under stationary conditions due to the rapid utilization of substrate but the yield of gallic acid is low due to the oxidation of gallic acid to propyl gallate, pyrogallol or other oxides. On the other hand, in the stationary MSSF condition there is a steady utilization of substrate by tannase giving higher tannase production and subsequently more gallic acid production.

Based on 58% tannin content in *C. digyna* seed cover powder, gallic acid recovered in MSSF was 90.9% while in SSF it was 30.8% and in SmF it was 27.5%. The yield of gallic acid from

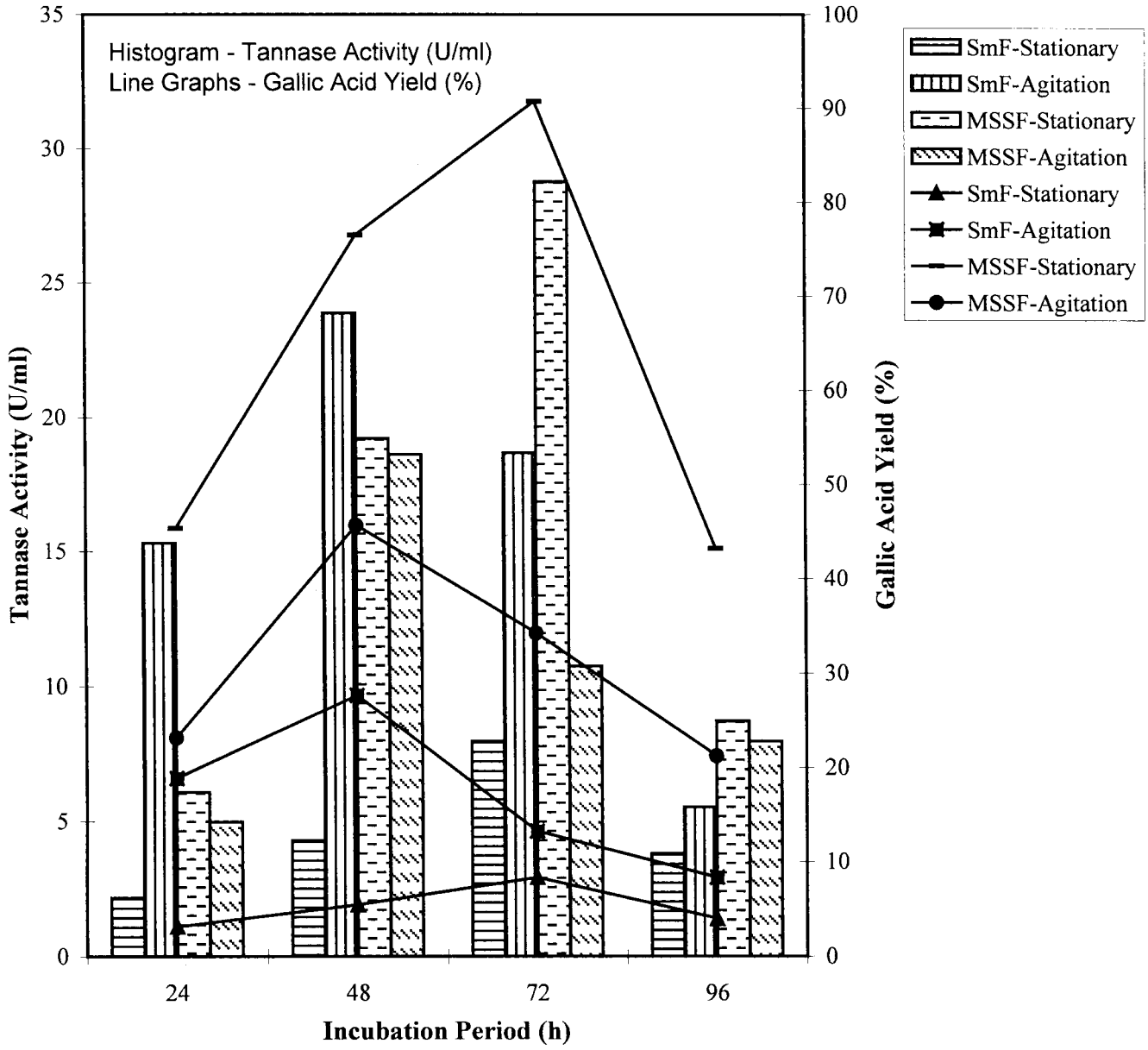

Figure 7 Effect of fermentation condition on tannase production SmF and MSSF conditions at a constant incubation period (48 h SmF, 72 h SSF and MSSF), substrate content (2 g SSF, 10% (w/v) SmF, 20 g MSSF), pH (4.5 SSF and MSSF, 5.0 SmF), temperature (32°C SSF and MSSF, 37°C SmF), solid:liquid ratio (1:1 SSF, 0.4:1 SmF and MSSF), relative humidity (93% SSF and MSSF), bed height (0.15 cm SSF, 1.5 cm MSSF).

tannic acid in free cell culture was 83.5% and 78.5% with immobilised cells [13]. Gallic acid obtained from sumac tannin containing 10% tannin was 9.75% under SmF conditions [15]. Gallic acid has also been produced from tara tannins using *A. niger* [14].

In conclusion, a higher yield of gallic acid can be obtained under MSSF conditions compared to SSF and SmF conditions. This can be attributed to the salient features of the GROWTEK bioreactor. As most of the biochemicals are heat sensitive, this bioreactor can overcome the problem of heat generated during the conventional SSF process due to the continuous contact of the liquid in the vessel with the solid substrate on the float.

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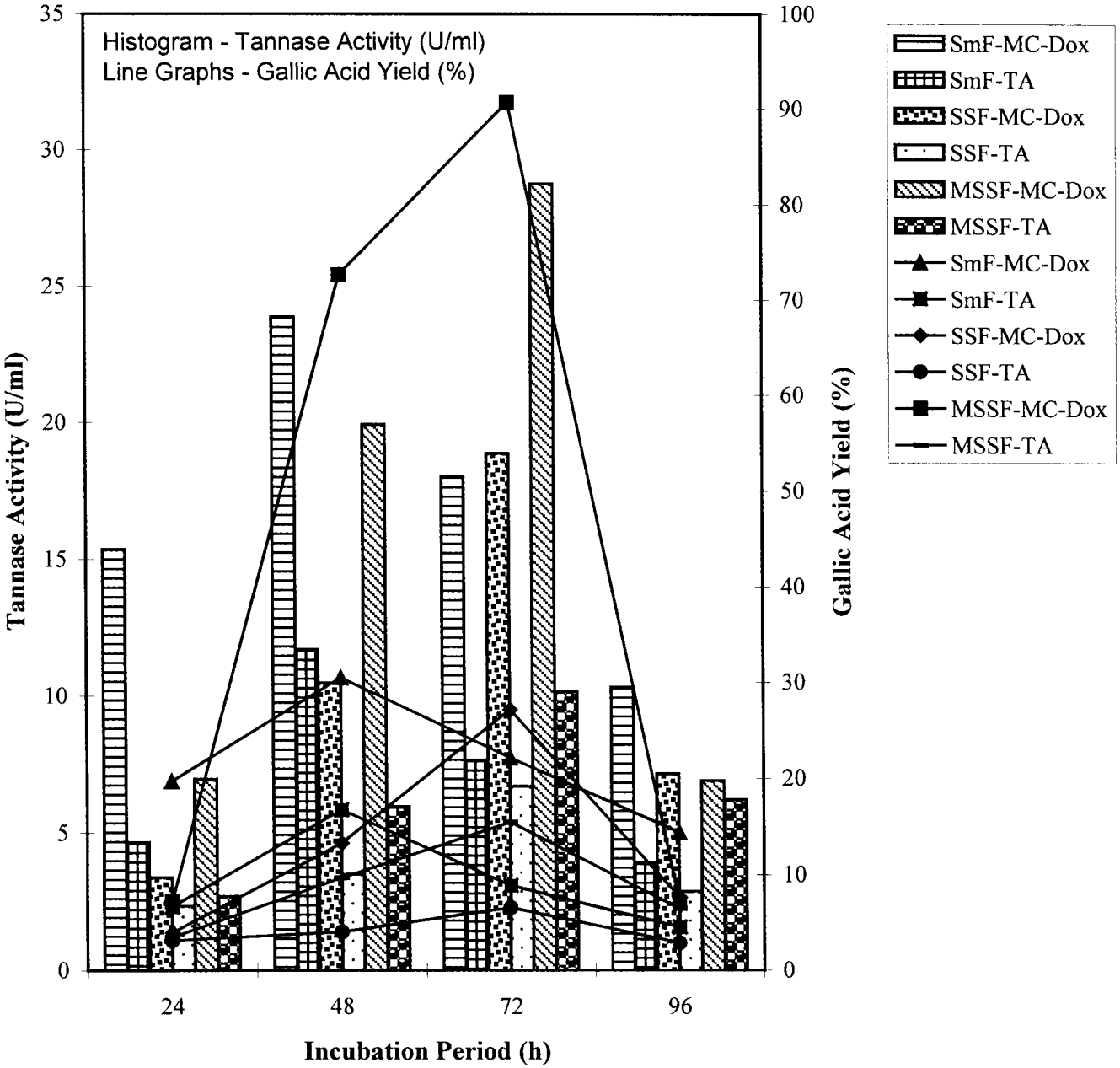

Figure 8 Effect of media on tannase production under SSF, SmF and MSSF conditions at a constant incubation period (48 h SmF, 72 h SSF and MSSF), substrate content (2 g SSF, 10% (w/v) SmF, 20 g MSSF), pH (4.5 SSF and MSSF, 5.0 SmF), temperature (32°C SSF and MSSF, 37°C SmF) solid:liquid ratio (1:1 for SSF, 0.4:1 SmF and MSSF), relative humidity (93% SSF and MSSF), bed height (0.15 cm SSF and 1.5 cm for MSSF).

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