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Full Length Research Paper

Isolation of gallic acid-producing microorganisms and their use in the production of gallic acid from gall nuts and sumac

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A total number of eighty gallic acid producing strains were isolated from forest soil or plant samples. Among these strains, thirteen isolates were selected for gallic acid production and these isolates were *Aspergillus niger* 1, *A. niger* 2, *A. niger* 3, *Penicillium canescens* (3), *P. frequentans* (2), *P. spinulosum* (2), *P. purpurogenum* (2), and *P. zacinthae*. By using eight of these strains and reference strain of *A. niger* NRRL 321, the production of gallic acid from oak tree (*Quercus infectoria*) gall nuts or sumac (*Rhus coriaria*) leaves were investigated. Maximum gallic acid yields from gall nuts were obtained for *A. niger* 3 (91.3%) and *P. spinulosum* (93.2%). In the case of sumac leaves, the reference strain *A. niger* NRRL 321 (46.1%) and *P. zacinthae* (48.2%) gave the highest gallic acid yields. To date, this study is the first report on production of gallic acid by these newly isolated *Penicillium* strains. Particularly, *A. niger* 3, *P. spinulosum*, *P. purpurogenum* and *P. canescens* may be used not only for gallic acid but also tannase production from tannin rich plant materials such as gall nuts. Their high yields and short incubation periods are also remarkable.

Key words: Gallic acid, gall nuts, gallotannin, sumac, tannase.

INTRODUCTION

Tannins are polyphenolic secondary metabolites of plants, which form hydrogen bonds in solutions, resulting in the formation of tannin-protein complexes (Sharma et al., 1999). They are found in a large array of herbaceous and woody plants and their molecular weights range from 500 to 3000 g mole^{-1} (Scalbert, 1991; Chamkha et al., 2002). Two groups of tannins are distinguished according to their structures: hydrolyzable and condensed ones (Regerat et al., 1989; Chamkha et al., 2002; Huang et al., 2005). Hydrolyzable tannins are composed of esters of gallic acid (gallotannins) or ellagic acid (ellagitannins) with a sugar core which is usually glucose (Bhat et al., 1998). They can occur in wood, bark, leaves, fruits and galls (Mueller-Harvey, 2001). Major commercial hydrolyzable tannin sources are Chinese gall (*Rhus semialata*), sumac (*Rhus coriaria*), Turkish gall (*Quercus infectoria*), tara (*Caesalpinia spinosa*), myrobalan nuts (*Terminalia chebula*), and chestnut (*Castanea sativa*) (Bhat et al., 1998). Hydrolyzable tannins are readily hydrolyzed chemically by acidification or biologically by an enzyme known as tannase (Tannin acyl hydrolase, EC 3.1.1.20) (Barthomeuf et al., 1994; Chamkha et al., 2002). Tannase catalyzes the hydrolysis of ester and depside linkages in hydrolyzable tannins like tannic acid releasing glucose and gallic acid (Mondal et al., 2000; Sharma et al., 2000; Batra and Saxena, 2005; Mahapatra et al., 2005; Sabu et al., 2005). Tannase is an industrially important enzyme and extensively used in the manufacture of gallic acid from gallotannin and instant tea, in the clarification of coffee and flavoured soft drinks, in the mediarization of wine and fruit juices, and stabilization of grape wine (Bajpai and Patil, 1997; Seth and Chand, 2000; Aguilar et al., 2001a; Aguilar et al., 2001b; Mondal et al., 2001a; Mondal et al., 2001b). It is also used as a sensitive analytical probe for determining the structure of naturally occurring

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gallic acid esters (Mondal and Pati, 2000; Seth and Chand, 2000). A potential use of tannase is the treatment of wastewater contaminated with polyphenolic compounds such as tannins (Aguilar et al., 2001a). Tannase has been characterized from strains of *Aspergillus* and *Penicillium* (Kar et al., 2003). In addition a number of microorganisms including bacteria (Deschamps et al., 1983; Deschamps and Lebeault, 1984; Osawa et al., 2000; Ayed and Hamdi, 2002; Nishitani and Osawa, 2003) and yeasts (Aoki et al., 1976; Bhat et al., 1998) also have been reported to produce tannase.

The enzymatic product gallic acid (3,4,5-trihydroxybenzoic acid) is a phenolic compound and finds applications in various fields. Its major application area is to manufacture trimethoprim (TMP) which is an antibacterial agent used in combination with sulphonamide (Hadi et al., 1994; Kar et al., 1999; Kar and Banerjee, 2000). It is also used in leather industry, in manufacturing gallic acid esters, such as propyl gallate, which is widely used as food antioxidant, in the manufacture of pyrogallol, and as a photosensitive resin in semiconductor production (Kar et al., 1999; Mondal and Pati, 2000; Banerjee et al., 2001; Mondal et al., 2001b). Pyrogallol is used in staining fur, leather and hair, and also as a photographic developper (Kar et al., 1999).

Various groups have reported the gallic acid production from myrobalan (Mukherjee and Banerjee, 2004), tara (Pourrat et al., 1985), sumac (Pourrat et al., 1987), gall nuts (Regerat et al., 1989), Chinese tannins (Kar et al., 1999), teri pod (*Caesalpinia digyna*) (Kar et al., 1999; Mukherjee and Banerjee, 2004) and sake cake (Kawakubo et al., 1993). In this study, we have screened the gallic acid-producing microorganisms, identified isolated microorganisms, and investigated gallic acid production from Turkish gall nuts and sumac leaves.

MATERIALS AND METHODS

Isolation and identification of gallic acid producing microorganisms

A number of gallic acid producing microorganisms was isolated according to Kawakubo et al. (1991, 1993) from 30 different soil samples from forest and 5 plant in basal medium with the following composition (w/v): gall nuts powder or glucose 0.5%, NH_4NO_3 0.12%, $MgSO_4.7H_2O$ 0.05%, KCl 0.01%, and KH_2PO_4 0.1%. The plant samples (white gall nut, black gall nut, valonia (*Quercus macrolepis*), sumac and acacia (*Robinia pseudoacacia*) leaves) were collected from Şabanözü-Çankırı, Mihalıççık-Eskişehir, Anadolu University-Yunus Emre campus, Gaziantep districts and soil samples were collected from Yeşilyurt-Tokat, Mihalıççık-Eskişehir and Bozdağ-Eskişehir districts in Turkey. The soil samples (about 2 - 10 cm of depth) were collected with a sterilized spatula from near the oak trees. The plant and soil samples were placed in sterile plastic bags, closed tightly and stored at 4°C until analysis. The pH of the medium was adjusted to 4.0, 5.0, 6.0, or 7.0. The medium was inoculated with diluted soil and plant samples and cultured at 30°C for 7 to 10 days. Isolation of microorganisms was carried out on the basal medium plates containing 0.5% gall nuts powder and 3% agar. Then these isolates were cultured in the basal medium containing 0.5% gall nuts powder, at 30°C for 7 to 10 days. Analysis of gallic acid was done by color reaction with 1% $FeCl_3$ solution and thirteen strains showing a good color reaction with $FeCl_3$ (black brown, black green, or black blue) were selected (Kawakubo et al., 1991; Kawakubo et al., 1993). Each isolate was subsequently grown on Potato Dextrose agar (PDA) plates for 7 to 10 days at room temperature and stored at 4°C on agar slants for further work.

Fungi were identified to genus level according to Barnett and Hunter (1999). The isolates were identified to species level according to various mycological references as below: *Penicillium* species were grown on 5 different media according to Pitt (2000). Cultures were inoculated in 3 points onto Czapek Yeast Extract agar (CYA) and incubated at 3 different temperatures (5, 25 and 37°C) for 7 days in the dark. In addition, Czapek-Dox agar (CDA), Malt Extract agar (MEA), Neutral Creatine Sucrose agar (CSN) and 25% Glycerol Nitrate agar (G25N) were used for the cultivation of *Penicillium* species (at 25°C, for 7 days) (Raper and Thom, 1949; Pitt, 2000). *Aspergillus* species were identified according to Raper and Fennell (1965), Hasenekoğlu (1991) and Klich (2002). Therefore, MEA, CDA, CYA with 20% sucrose (CY20S), CYA (at 25 and 37°C) medium were prepared and *Aspergillus* culture was inoculated into each medium and incubated at 25°C (except CYA37), for 7 days. All names of the identified species and authors were cited according to Kirk and Ansell (1992).

In addition to above isolates, *Aspergillus niger* NRRL 321, obtained from the culture collection of the United States Department of Agriculture, Agricultural Research Service, National Center for Agricultural Utilization Research, was used as a reference strain.

Raw materials

Gall nuts and sumac leaves were used as raw materials in the biotransformation studies. They were dried at 50°C in an oven, finely ground in a grinder, and stored in a dry place at room temperature.

Ground gall nuts (1%, w/v) or sumac leaves (1%, w/v) were extracted for tannins with hot water. This treatment for 1 h at 95 - 100°C removed all tannins (Pourrat et al., 1985; Pourrat et al., 1987; Regerat et al., 1989). The substrates were inoculated directly without prior filtration.

Inoculum

Inoculum was prepared using PDA slants. The slants were inoculated and incubated at room temperature for 7 to 10 days. Spores were then scraped into 0.5% Tween-80 and counted using a Thoma slide.

Fermentation

For gallic acid production, growth of the fungal strains was carried out in 150 ml Erlenmeyer flasks containing 50 ml extracted plant materials (pH 5.8 - 6.0). These flask contents were inoculated with 3.7×10^6 spores/ml of the medium and incubated for 72 h at 30°C in an incubator shaker at 120 rpm. Gallic acid production was measured at various times (9, 24, 33, 48, 57 and 72 h) during incubation period. Control samples without inocula were used and all experiments were carried out in duplicates.

Assay of gallic acid

The breakdown of the tannins was monitored during fermentation by assay of released gallic acid (Osawa and Walsh, 1993). After 0,

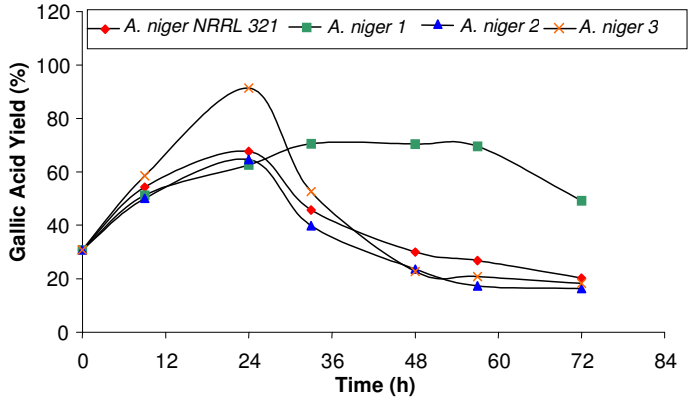

Figure 1. Time course of release of gallic acid production using gall nuts.

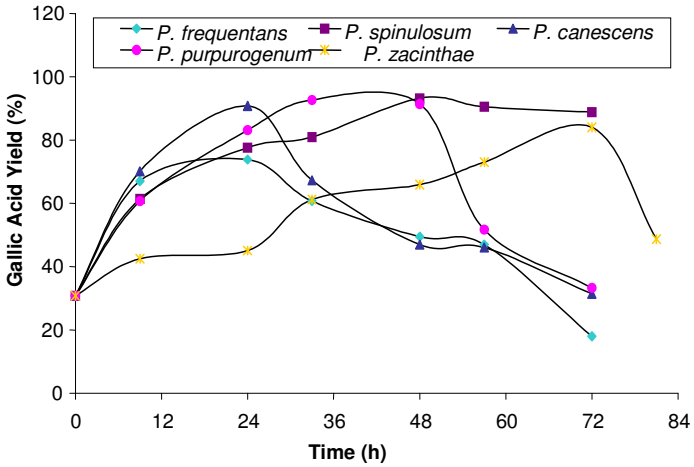

Figure 2. Time course of release of gallic acid production using gall nuts.

9, 24, 33, 48, 57, and 72 h of fermentation, 2 ml of the suspension was aseptically removed, 2 - 3 drop of sodium azide solution (1%) was added to samples in order to stop the activity of tannase, and filtered. The samples were alkalinized with equal amount of saturated $NaHCO_3$ solution (pH 8.6) and then exposed to the atmosphere at room temperature (23°C) for 1 h which resulted in green to brown coloration of the medium. Absorbance at 440 nm was read in a spectrophotometer (UV-VIS Scanning Spectrophotometer Shimadzu UV-2101 PC) and the amount of gallic acid in the medium was determined using a calibration curve. Linear regression analyses were applied and gallic acid concentration as molar (M) was calculated as in the following regression equation:

$$A = 1090\ C(M) + 0.00129$$

where, A is absorbance; 1090 is slope; C(M) is molar concentration of gallic acid; 0.00129 is intercept.

All gallic acid assays were performed in duplicate and with appropriate blank. Gallic acid yields were calculated as percent yield using molar concentration of the gallic acid with respect to the weight of raw material.

RESULTS AND DISCUSSION

Gallic acid producing microorganisms were not isolated in glucose containing basal medium. In contrast, eighty strains were isolated from 30 different forest soil samples and 5 plant samples when the basal medium containing gall nuts powder was used. Among them, thirteen strains giving a good color reaction with $FeCl_3$ were selected and three of them were identified as *A. niger* which was tentatively named *A. niger* 1, *A. niger* 2 and *A. niger* 3. Rest of the strains were identified as 3 strains of *Penicillium canescens*, 2 strains of *Penicillium frequentans*, 2 strains of *Penicillium spinulosum*, 2 strains of *Penicillium purpurogenum* and 1 strain of *Penicillium zacinthae*.

Kawakubo et al. (1991) reported screening results of gallic acid producing microorganisms from 660 soil samples by using basal medium containing glucose. In that study, ten strains giving a color reaction with $FeCl_3$ were isolated and three of them were gallic acid producing microorganisms belonging to the genus *Penicillium*. Shortly after that report, the same group (Kawakubo et al., 1993) reported the use of a sake cake medium for screening gallic acid producing microorganisms and identified *Aspergillus terreus* S-4 as a gallic acid producing microorganisms and provided some culture conditions for the acid production. As given above, we have isolated three *A. niger* and five *Penicillium* strains from our soil and plant samples.

The result of the production of gallic acid from gall nuts by *A. niger* strains are plotted in Figure 1. *A. niger* 3 gave maximum gallic acid yield as high as 91.3% at 24 h of the fermentation time. Regerat et al. (1989) reported a 40.5% yield of gallic acid production from gall nuts by *A. niger* with a fermentation time of 24 h. Among our *Penicillium* strains, maximum amount of gallic acid was produced by *P. spinulosum* at a yield of 93.2% at 48 h (Figure 2). *P. canescens* (90.8%) and *P. purpurogenum* (92.7%) were also good gallic acid producer strains (Figure 2). Maximum gallic acid yields from these strains were obtained shortly incubation periods at 24 and 33 h, respectively.

Among *Aspergillus*, the reference strain *A. niger* NRRL 321 gave the highest gallic acid yield from sumac leaves (46.1%) after 9 h of fermentation time (Figure 3). Pourrat et al. (1987) reported maximum gallic acid yield as 9.75% from sumac leaves when tannase-producing strain *A. niger* was used. Among our *Penicillium* strains, maximum gallic acid from sumac leaves was produced from *P. zacinthae* at a yield of 48.2% yield after 24 h fermentation time (Figure 4). In the literature, Pourrat et al. (1985) obtained gallic acid from tara tannin at 30% with respect to the weight of raw material. Kar and Banerjee (2000) were able to produce gallic acid from *C. digyna* seed cover tannins using *Rhizopus oryzae* (RO IITKGP RB-13,

Table 1. Microorganisms, substrates and yields for gallic acid production in the scientific publications.

Microorganism	Substrate	Incubation period (h)	Gallic acid yield (%)	Reference
Aspergillus niger	Tara fruit pods	45	30	Pourrat et al. (1985)
Aspergillus niger	Sumac leaves	40	9.75	Pourrat et al. (1987)
Aspergillus niger	Gall nuts	24	40.5	Regerat et al. (1989)
Rhizopus oryzae (free cells)	2% Tannic acid in media	96	83.5	Misro et al. (1997)
Rhizopus oryzae (immobilized cells)	2% Tannic acid in media	96	78.5	Misro et al. (1997)
Rhizopus oryzae	Teri pod cover	72	90.9	Kar et al. (1999); Kar et al. (2002)
Rhizopus oryzae	Myrobalan and teri pod cover (mixed substrates)	60	85.67	Mukherjee and Banerjee (2004)
Aspergillus foetidus	Myrobalan and teri pod cover (mixed substrates)	72	90.48	Mukherjee and Banerjee (2004)
Rhizopus oryzae and *Aspergillus foetidus* (co-culture)	Myrobalan and teri pod cover (mixed substrates)	48	94.8	Banerjee et al. (2005)

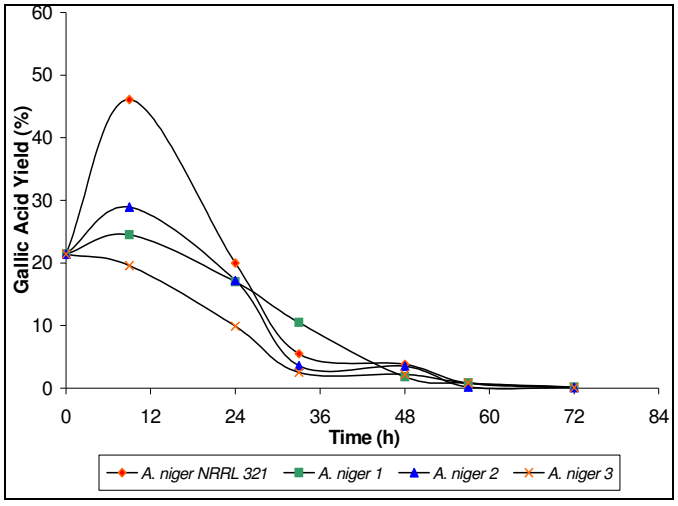

Figure 3. Time course of release of gallic acid production using sumac leaves.

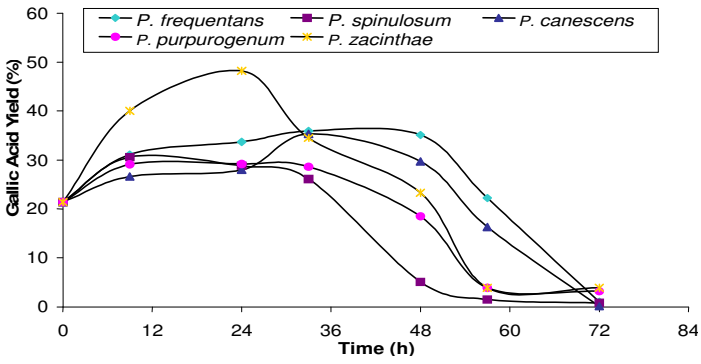

Figure 4. Time course of release of gallic acid production using sumac leaves.

NRRL 21498).

For different species of *Aspergillus* and *Penicillium*, Figures 1 - 4 showed that the initial increase in gallic acid production followed the by a decrease. The different reasons for observes decline in gallic acid production reported by other workers. Mahadevan and Sivaswamy (1985) explained that *A. niger* could degrade gallic acid and intermediates of this degradation could be *cis*-aconitic, α-ketoglutaric and citric acids. In addition, Saxena et al. (1995) determined species of *Aspergillus* and *Penicillium* could utilize catechin, gallotannin and gallic acid as carbon sources. Another reason for the decrease in gallic acid production, gallic acid itself acts as a competitive inhibitor (Kar et al., 1999; Kar and Banerjee, 2000).

The incubation period is very important as it decides the economics of a plant (Misro et al., 1997). At the 9, 24 or 48 h of incubation, the yield of gallic acid was maximum (Figures 1 - 4). In a number of fungal systems, tannins have been degraded rapidly in the presence of other metabolisable substance (Bhat et al., 1998).

Although recent studies have reported using several fungal species for gallic acid production (Table 1), this is the first study reporting gallic acid producing capabilities of *P. canescens*, *P. frequentans*, *P. spinulosum*, *P. purpurogenum* and *P. zacinthae*. These newly isolated *Penicillium* strains are very suitable for gallic acid production because they can grow easily and produce a huge amount of gallic acid within a short incubation periods. *Penicillium* strains in this study are not only a potent new group of gallic acid producers but also new sources of tannase for literature except *P. frequentans* (Table 2).

These fungal strains, especially *A. niger* 3, *P. spinulosum*, *P. purpurogenum* and *P. canescens* may be employed for gallic acid and tannase production from substrates containing high hydrolyzable tannins in large-scale

Table 2. Tannase sources from the genera *Penicillium*.

Microorganism	Reference
Penicillium notatum	Ganga et al. (1977)
Penicillium islandicum	Ganga et al. (1977)
Penicillium digitatum	Bradoo et al. (1996)
Penicillium acrellanum	Bradoo et al. (1996)
Penicillium carylophilum	Bradoo et al. (1996)
Penicillium chrysogenum	Bradoo et al. (1996)
Penicillium citrinum	Bradoo et al. (1996)
Penicillium charlessi	Bradoo et al. (1996); Batra and Saxena (2005)
Penicillium frequentans	Van de Lagemaat et al. (2000)
Penicillium variable	Saxena and Saxena (2004); Batra and Saxena (2005); Sharma et al. (2008)
Penicillium glaucum	Lekha and Lonsane (1997)
Penicillium crustosum	Batra and Saxena (2005)
Penicillium restrictum	Batra and Saxena (2005)
Penicillium glabrum	Van de Lagemaat and Pyle (2005)

applications such as pharmaceutical, food and feed and in the leather industry.

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