ABSTRACT

Biosynthesis of the industrially important enzyme tannase and gallic acid from a combination of tannin-rich substrates was carried out by modified solid-state fermentation (MSSF) in a bioreactor. The substrate was a mixture of powdered fruits of *Terminalia chebula* and *Caesalpinia digyna* pod cover powder. The two fungal cultures *Rhizopus oryzae* (RO IIT RB-13 NRRL 21498) and *Aspergillus foetidus* (GMRB013 MTCC 3557) isolated from the local soil of IIT Kharagpur Campus were used as inoculum. The mixing of the two materials showed the possibility of carrying out the process with combined substrates according to availability and economic convenience.

The optimum levels of the different process parameters for the maximum production of tannase and gallic acid were determined by varying the parameters one at a time. The fungal strain *Aspergillus foetidus* showed a marginally higher yield of tannase (36.4 U/ml) and gallic acid (90.48%) than *Rhizopus oryzae*, and it took a longer incubation period (72 h) for this maximal production. The highest yield of tannase (31.8 U/ml) and gallic acid (85.67%) was obtained after 60h in case of *Rhizopus oryzae*. The optimum initial pH of the fermentation was found to be 4.5 in case of *Rhizopus oryzae* and 5.0 for *Aspergillus foetidus*. The optimum levels of other parameters were 30 g of substrate, 3 ml of induced inoculum, a temperature of 30°C and 80% relative humidity. Collectively, the data reveal the potential of the MSSF process for the production of tannase and gallic acid from tannin-rich substrates with *R. oryzae* and with *A. foetidus*.

The two filamentous fungi, *R. oryzae* and *A. foetidus* flourished well together, complementing their requirements leading to a more efficient production of tannase and gallic acid than that produced by their individual cultures. Modified solid-state fermentation of the tannin rich mixed substrates by the co-culture method was carried out, yielding high amounts of tannase (41.3 U/ml) and gallic acid (94.8%). An optimum volume of 3 ml of induced inoculum of the two fungal cultures (in 1:1 ratio) was added to 35 g of mixed substrate on the mesh of the GROWTEK bioreactor. The optimum levels of the other process parameters for maximum tannase and gallic acid production by co-culture method were pH 5.0, an incubation period of 48 h at 30°C and 80% relative humidity.

The experimental data obtained were processed to obtain an empirical correlation for predicting the gallic acid production by the co-culture method through MSSF of tannin-rich substrates. The coefficient of correlation (r) was high (0.9591), indicating a very close agreement between the experimental and predicted values.

A further study of the optimum level of the process parameters was carried out by performing factorially designed experiments with simultaneous variations of the parameters. The interaction effects of the six influencing parameters i.e., temperature, relative humidity, incubation period, pH, inoculum volume and substrate amount on gallic acid production by co-culture method were thus investigated. The methodology adopted for the purpose was the Evolutionary Operation (EVOP)-factorial design technique which combines the factorial method for designing experiments with the EVOP methodology for analyzing the experimental results systematically and arriving at conclusions according to its decision-making procedure. From the results it was found that the

best combinations of the process parameters at the optimum levels were 30°C temperature, 80% relative humidity, 48 h incubation period, 3 ml of induced inoculum, 35 g mixed substrate and pH 5 resulting in a gallic acid yield of 94.8% under MSSF condition.

Purification of the tannase obtained from *R. oryzae*, *A. foetidus* and their co-culture by MSSF has been done following the procedures of solvent precipitation and DEAE-sephadex column chromatography. Studies on kinetics revealed an optimum temperature of 40°C for activity of tannase obtained from all the three sources, which was above the laboratory ambient temperature. This is advantageous as there is less chance of enzyme denaturation. The optimum incubation period required by *Rhizopus* tannase was 4 min, and that for tannase obtained from both *Aspergillus* and the co-culture was 5 min. The pH optima (4.5 for *R. oryzae* tannase and 5.0 for the other two) were in the acidic range. The optimum initial substrate concentration was bit lesser in case of *Rhizopus* tannase (0.35 mg/ml) than the tannases obtained from *Aspergillus* and the co-culture (0.42 mg/ml). The optimum initial enzyme concentration required was also lesser for *Rhizopus* tannase (0.08 v/v) compared to that required for tannase obtained from *Aspergillus* and the co-culture (0.12v/v). The half-life period, thermal stability were determined, and the effects of metal ions, surfactants, chelator, denaturant, and inhibitor on the activity of tannase obtained from *Rhizopus oryzae*, *Aspergillus foetidus* and their co-culture were also studied, which were found to vary. Among metal ions, only Mg^{++} enhanced activity of *R. oryzae* and co-culture tannase, while most other metal ions (Fe^{+++}, Ba^{++}, Ca^{++}, Zn^{++}, Mn^{++}, Hg^{++}, Cu^{++}) have inhibited activity of tannase from all the sources. Surfactants (like SLS, Tween 40, Tween 80, Tween 60, Triton X-100) have inhibited tannase activity to different extents. Urea induced activity in case of *R.*

oryzae and co-culture tannase at a low concentration and inhibited it at higher concentrations. Among chelators, EDTA caused the maximum inhibitory effect on *R. oryzae* tannase, compared to the other two, while 1,10-*o*-phenanthroline caused a greater inhibition of *A. foetidus* tannase. The inhibitor, β-mercaptoethanol caused a greater inhibition of *Aspergillus* tannase. The co-culture tannase showed highest catalytic activity followed by the *Aspergillus* tannase and then by the *Rhizopus* tannase when kinetic studies were carried out for the characterization of the enzymes. The half-life period at the optimum temperature (40°C) and the temperature stability of the co-culture tannase were highest followed by *Aspergillus* and then *Rhizopus* tannase.

Key words: Tannase, gallic acid, filamentous fungi, *Rhizopus oryzae*, *Aspergillus foetidus*, *Terminalia chebula*, *Caesalpinia digyna*, modified solid-state fermentation, bioreactor, single culture, co-culture, empirical correlation, EVOP-FD design, purification, characterization.