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ORIGINAL PAPER

Optimization of production of caffeine demethylase by *Pseudomonas* sp. in a bioreactor

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Abstract The effect of pH, aeration rate, and agitation rate on specific productivity of caffeine demethylase from *Pseudomonas* sp. was studied in a bioreactor. Maximum specific productivity of caffeine demethylase of 2,214 U g cell dry weight^{-1} h^{-1} was obtained at 0.27 vvm, 700 rpm, and pH 7.0. Under these conditions, volumetric oxygen transfer coefficient was 74.2 h^{-1}, indicating that caffeine demethylase production by *Pseudomonas* sp. was highly oxygen-dependent. Different metabolite formation at different agitation and aeration rates can be used as a strategy for recovery of pharmaceutically important metabolites from caffeine by manipulation of conditions in a bacterial culture. This is the first report on production of high levels of caffeine demethylase in bioreactors.

Keywords Aeration rate · Agitation rate · Bioreactor · Caffeine degradation · Caffeine demethylase · *Pseudomonas* sp

Introduction

The deleterious effect of caffeine is apparent from the numerous reports on the association of habitual caffeine intake with adverse effects on the cardiovascular system and health of women [4, 20]. Caffeine removal from coffee and tea byproducts is also important, keeping in mind the toxicity of caffeine to microflora of water bodies and soil around coffee-processing regions and hence the environment [5]. Decaffeination is, therefore, an important step in coffee and tea processing. In this regard, biodecaffeination using microbes or microbial enzymes, although not commercialized, has been considered more suitable than currently used physical and chemical methods of decaffeination as a result of being specific, economical, and environmentally friendly [16]. Concerns would be issues arising because of the direct use of bacterial cells or crude enzyme preparations for decaffeination of caffeinated food products, because of possible bacterial contamination and impurity of crude enzymes. This can be avoided by using specific caffeine-degrading enzymes in the pure form. Difficulties arising as a result of diffusion of enzyme(s) during decaffeination of caffeinated plant products could be yet another consideration. Nevertheless, decaffeination by enzymatic methods is, in particular, more suitable for food products where caffeine is removed selectively keeping other components, for example flavor and aroma-imparting constituents, unaltered.

Several bacteria and fungi, viz. *Pseudomonas*, *Klebsiella*, *Alcaligenes*, *Penicillium*, and *Aperigillus*, capable of utilizing caffeine as the sole carbon and nitrogen source have been isolated [2, 26, 29]. However, the inability to tolerate high concentrations and degrade caffeine at faster rates necessitates the need for a better strain. Studies of the enzymes involved in caffeine degradation are also not explicit, with the exception of caffeine oxidase, which has been purified and characterized [24, 29]. A few studies have shown there might be separate enzymes, acting separately [1, 14] or as a complex [3, 12], which bring about specific demethylations in *Pseudomonas* sp.

In this respect, a previously isolated strain of *Pseudomonas* closely resembling *Pseudomonas putida* was capable of utilizing caffeine as sole carbon and nitrogen source [9, 17]. The strain exhibited substrate inhibition

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kinetics when grown on caffeine as sole source of carbon and nitrogen and the minimum inhibitory concentration of caffeine was 20 g l^{-1} [18]. It could completely degrade caffeine at an initial concentration of 5 g l^{-1} at a rate of 0.1 g l^{-1} h^{-1}, which was further increased to 0.29 g l^{-1} h^{-1} by optimization of medium components and physical conditions [10, 11], thus proving to be better than other caffeine-degrading strains isolated so far. The degradation of caffeine by this strain was via a demethylation pathway, as indicated by metabolites formed by growing culture [9] and by enzymatic assay [12]. In this study, batch reactor experiments were performed to investigate the effect of pH, agitation, and aeration rates on caffeine demethylase production by *Pseudomonas* sp. The findings shall be useful in defining conditions and developing strategies for production of a caffeine-degrading enzyme in *Pseudomonas* sp.

Materials and methods

Chemicals

Pure caffeine (1,3,7-trimethylxanthine) was obtained from Merck. Theobromine (3,7-dimethylxanthine), paraxanthine (1,7-dimethylxanthine), theophylline(1,3-dimethylxanthine), 7-methylxanthine, NADH, and dithiothreitol (DTT) were obtained from Sigma. All other reagents were of analytical grade and procured in India.

Bacterial strain and medium

Pseudomonas sp. NCIM 5235 was maintained on CAS agar medium of composition: Na_2HPO_4, 0.12 g l^{-1}; KH_2PO_4, 1.3 g l^{-1}; $CaCl_2$, 0.3 g l^{-1}; $MgSO_4.7H_2O$, 0.3 g l^{-1}, sucrose, 5 g l^{-1}; caffeine, 1.2 g l^{-1}; and agar, 25 g l^{-1} (pH 6.0). The strain was sub-cultured every alternate day. Reactor studies were performed with optimized CAS medium [10] of composition: Na_2HPO_4, 0.352 g l^{-1}; KH_2PO_4, 3.4 g l^{-1}; $CaCl_2$, 0.3 g l^{-1}; $MgSO_4.7H_2O$, 0.3 g l^{-1}; sucrose, 5 g l^{-1}; caffeine, 6.4 g l^{-1}; and Fe^{2+}, 0.075% (w/v).

Fermentation runs

To study the effect of pH, aeration, and agitation on caffeine demethylase production, experiments were performed in a stirred 7.5 l bioreactor (Bioflo 110; New Brunswick Scientific, USA) with 3.75 l optimized CAS medium as working volume. The medium was inoculated with 6% (v/v) (OD_{600nm} ~1.4) of cells grown at 30°C for 2.5 h in nutrient broth medium. Calibration of the percentage of atmospheric oxygen was performed by sparging air into the medium until saturation (100%) and by sparging nitrogen (0%) after sterilization. Reactor studies were carried out at pH ranging from 5.0 to 9.0 to discover the optimum pH condition. To study the effect of agitation, experiments were carried out at 400, 500, 700, and 800 rpm. The aeration rates were set at 0.27, 0.4, 0.5, and 1 vvm to study the effect of aeration. Samples were drawn at regular intervals and analyzed for cell growth, caffeine content, metabolite formation, reducing sugars, and enzyme activity.

Assay for caffeine demethylase and other methylxanthine degrading activity

Pseudomonas sp. cells were harvested and washed three times with 50 mM potassium phosphate buffer (pH 8.0). The cells were then suspended in lysis buffer (50 mM phosphate buffer (pH 8.0) containing 1 mM EDTA, 1 mM DTT, 20% glycerol and 10% ethanol) with cell mass-to-buffer volume ratio of 1:2 (w/v). This step was carried out immediately after the last wash with 50 mM potassium phosphate buffer (pH 8.0) as mentioned above. The cells were then disrupted by sonication over ice (four cycles of 2 min each with adequate cooling between cycles). The cell debris was separated by centrifugation at 20,000×*g* and 4°C for 30 min. The supernatant was treated as the crude enzyme extract and was immediately used in the assay.

Enzyme activities were measured at 30°C in reaction mixture consisting of 7.5 mM caffeine in 50 mM potassium phosphate buffer (pH 8.0), and 1 mM NADH. Reaction was initiated by adding cell-free lysate containing 50 μg protein and reaction was stopped after 10 min by addition of 10% (w/v) trichloroacetic acid (TCA). Reaction carried out with enzyme inactivated with TCA prior to incubation served as blank for the assay. The reaction mixture was then centrifuged at 20,000×*g* and 4°C for 15 min and the supernatant was analyzed by HPLC. A similar procedure was followed for other methylxanthines, caffeine being replaced by the other methylxanthines (1 mM). One unit of enzyme activity (U) was defined as the number of micromoles of substrate (caffeine or other methylxanthines) degraded per minute of reaction. All assays were repeated at least twice, in duplicate, and the results presented are the average of four separate data values.

Analytical methods

Cell concentration was monitored by measuring optical density at 600 nm. Cell dry weight for *Pseudomonas* sp. was calculated from OD_{600nm} values (OD_{600nm} of 1 corresponds to 0.75 g l^{-1} cell dry weight). Sucrose was analyzed by the DNS method [28]. Protein was estimated by the method described by Lowry et al. [23]. Caffeine and other methylxanthines were estimated by RP-HPLC (Jasco

PU-2080 Plus equipment) using a HiQSil C-18 column with water–methanol 70:30 (v/v) as mobile phase at a flow rate of 1 ml min^{-1} and at 28°C. Pure caffeine and other methylxanthines (Sigma) at 2 g l^{-1} were used as standards. UV detection was at 254 nm [9].

Determination of K_La

K_La was determined in the culture medium by the gassing out method [32]. The medium was first degassed with nitrogen, followed by aeration and agitation at the conditions mentioned above. Absorption of oxygen was measured using a DO probe by recording the rising DO concentration as a function of time. The value of K_La is the slope of the semi-logarithmic plot of $(1 - pO_2)$ against time, in accordance with the equation:

$$\ln(1 - pO_2) = -K_L a * t + c \quad (1)$$

where c = constant, t = time in seconds, and pO_2 = percentage dissolved oxygen concentration in broth.

Results and discussion

Caffeine *N*-demethylases seem to be an important class of enzymes that are appropriate for biodecaffeination of caffeinated food products. This paper is the first report of the production of *N*-demethylases from caffeine degrading *Pseudomonas* sp. in a bioreactor and the effect of various reactor conditions affecting production of these enzymes by the bacterial strain.

Effect of controlled pH on growth, caffeine degradation, and enzyme production

The effect of pH on caffeine degradation in the bioreactor was studied at different pH (5.0, 6.0, 7.0, 8.0, and 9.0) at agitation and aeration rates of 500 rpm and 0.27 vvm, respectively. Growth was greatly reduced at pH 5.0 and 6.0 whereas a long lag phase was observed when the pH of the medium was 9.0 (Fig. 1a). This matched the DO profile for each run—oxygen consumption was lower at pH 5.0, 6.0, and 9.0 (Fig. 1b). Caffeine was more rapidly consumed at pH 7.0 than at other pH, and caffeine degradation rate and specific growth rate were maximum at pH 7.0 (Figs. 1c, e). However, sucrose was not at all consumed at all pH (data not shown), which is in agreement with previous reports that sucrose enhanced caffeine degradation without being utilized [19].

Earlier studies on *Pseudomonas* sp. in a shake flask have shown that degradation of caffeine was strongly affected by pH [17]. This was because of high rates of metabolite formation during active degradation of caffeine leading to accumulation of nitrogenous methylxanthines. This increased the alkalinity of the medium so much so that the pH of the medium went beyond 9.0, resulting in inhibition of bacterial growth and caffeine degradation [11]. In our bioreactor study, however, a long lag phase was observed when *Pseudomonas* sp. was grown at pH 9.0 but growth and caffeine degradation were not completely inhibited, as observed in shake flasks. This was probably because the pH was maintained at 9.0 in bioreactors compared to uncontrolled pH in shake flasks and consequential increase in pH beyond 9.0.

At the optimum pH (7.0), the maximum specific productivity for caffeine demethylase activity was 2300 U g cell dry $weight^{-1}$ h^{-1} (Fig. 1d). It was seen that the cell-free extract was active toward all the metabolites tested, further confirming the presence of a multi-enzyme caffeine catabolizing system in *Pseudomonas* sp., as reported earlier [3, 12]. However, the specific productivity was higher for caffeine than for the other substrates. This is in agreement with previous studies on this strain, which showed that the enzymes involved in caffeine degradation are inducible in nature [12]. Because the strain was cultivated in medium containing caffeine, the enzymes utilizing caffeine as substrate were expressed in higher amounts than the other enzymes.

Effect of agitation on growth, caffeine degradation and enzyme production

Agitation is an important reactor condition and is beneficial in enhancing the efficiency of substrate utilization by the enzyme and, in some cases, product formation. In other cases it may lead to deleterious effects such as rupture of cell walls and changes in cell morphology, because of shear stress [25]. Hence it was necessary to determine the best conditions of agitation at which production of caffeine demethylase was highest without affecting the cells. The effect of agitation on cell growth, caffeine degradation, and enzyme production by *Pseudomonas* sp. was studied by varying the agitation rates (400, 500, 700 and 800 rpm), at an aeration rate of 0.27 vvm and pH 7.0 for all the experiments. Maximum cell dry weight 0.85–1.0 g l^{-1} was attained when cells are grown at different agitation rates, but maximum cell growth was obtained in 18–20 h at 700–800 rpm (Fig. 2a). This can be attributed to the increase in dispersion of oxygen supplied through air at high agitation rates. Also, the consumption of oxygen was more rapid at 800 and 700 rpm than at 400 and 500 rpm (Figs. 1b, 2b), which can be attributed to insufficient mixing of the culture medium at lower agitation rates. Similar observation was found during curdlan production by *Agrobacterium* sp., where decreases in DO values corresponded to oxygen consumption and an increase in cell dry weight was

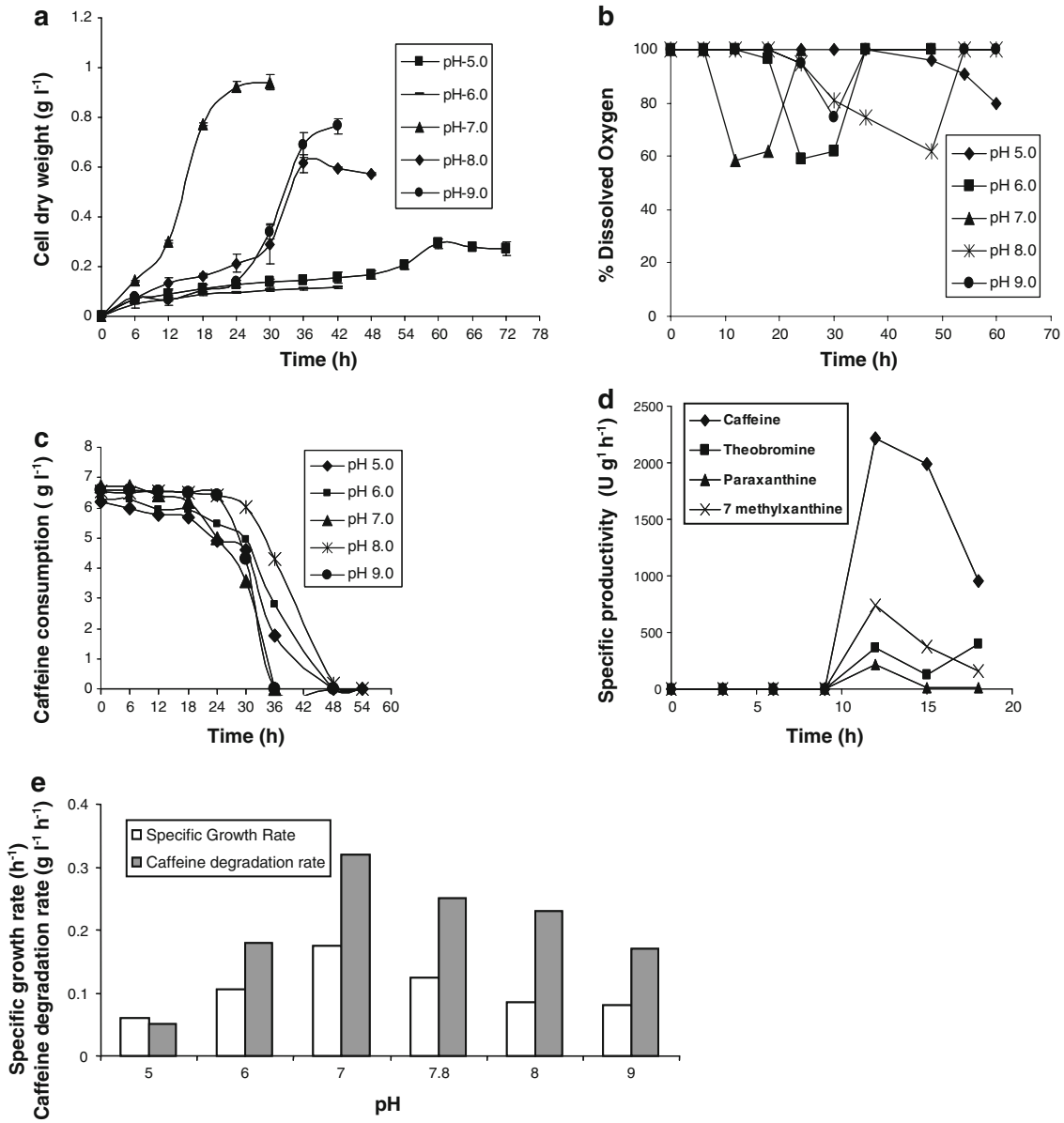

Fig. 1 Effect of pH on **a** cell growth, **b** % dissolved oxygen, **c** caffeine consumption, **d** specific enzyme productivity upon caffeine consumption at pH 7.0, and **e** specific growth rate and caffeine degradation rates of *Pseudomonas* sp. Batch reactor studies were carried out on 3.75 l optimized CAS medium as working volume, with agitation and aeration rates of 500 rpm and 0.27 vvm, respectively, at 30°C. Caffeine and metabolites were analyzed by RP-HPLC using a C-18 column and water–methanol 70:30 (v/v) as mobile phase

observed [22]. Similar results were also observed for nitrilase production by *Pseudomonas putida* in a bioreactor [30]. Caffeine consumption was 100% in 18 h when grown at 700 and 800 rpm and consumption was lower at 400 and 500 rpm, further confirming that caffeine degradation was strongly regulated at higher agitation rates (Figs. 1c, 2c). Consequently, enzyme productivity was maximum (2,314.3 U g cell dry weight^{-1} h^{-1}) at 10–15 h when grown at 700 rpm (i.e. K_La of 74.2 h^{-1}) (Fig. 2d). Hence 700 rpm was determined to be the optimum agitation rate.

The metabolite profile was studied at the log phase of growth of the strain, keeping the time point constant (15 h) to enable comparison of levels of different metabolites. Under lower agitation conditions, i.e. at 400 and 500 rpm, formation and accumulation of theobromine was favored, probably because of the lower agitation rate and, hence, subsequent interference with the activity of methylxanthine-degrading enzymes, as seen in a previous study [34]. Also, in comparison with other metabolites the amount of theobromine detected was highest for all conditions of

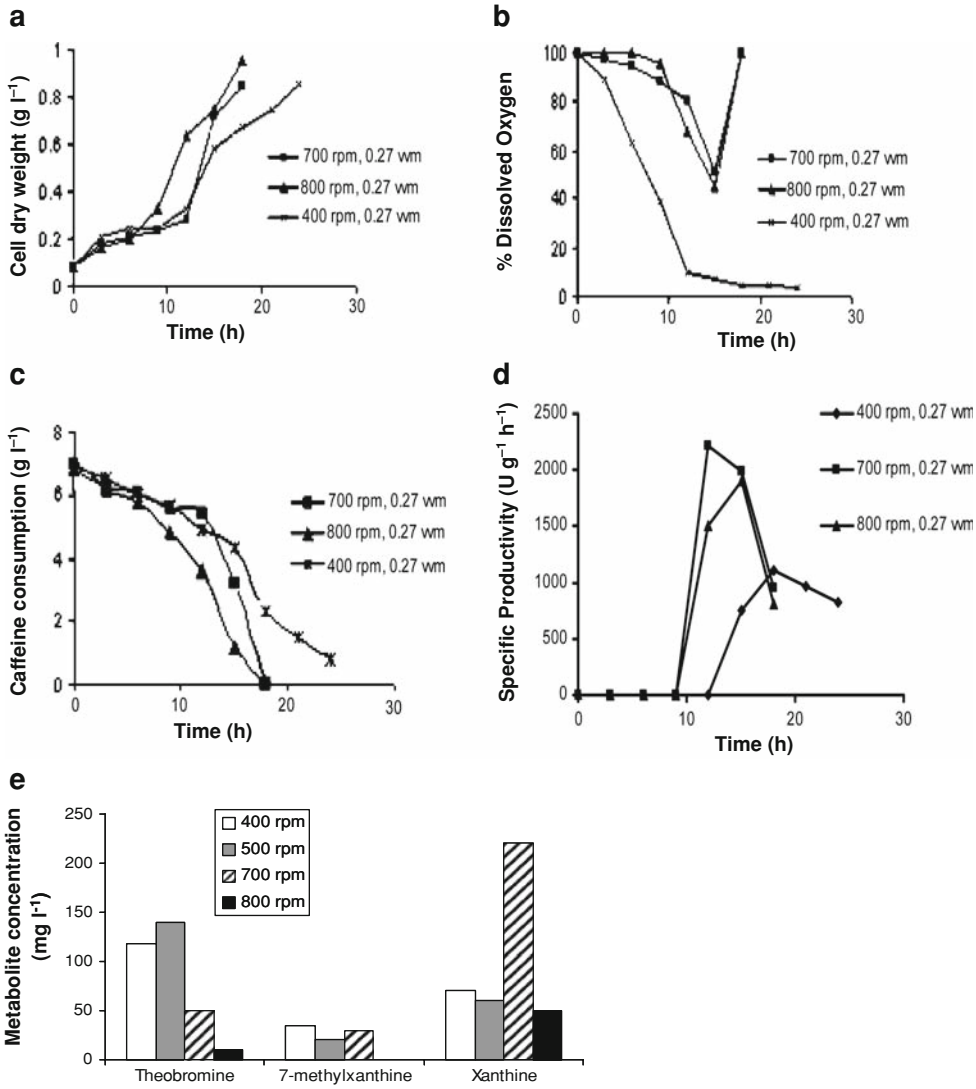

Fig. 2 Effect of agitation on **a** cell growth, **b** % dissolved oxygen, **c** caffeine consumption, **d** specific enzyme productivity, and **e** formation of metabolites in a bioreactor by *Pseudomonas* sp at 15 h of growth. Batch reactor studies were carried out at an aeration rate of 0.27 vvm and pH 7.0 at 30°C for all the experiments. Analysis of caffeine and metabolites was performed by RP-HPLC using a C-18 column and water–methanol 70:30 (v/v) as mobile phase

agitation except at 700 rpm where accumulation of xanthine (220 mg l^{-1}) was maximum (Fig. 2e).

Effect of aeration on growth, caffeine degradation, and enzyme production

Aeration level is one of the key conditions influencing dissolved oxygen concentration in the medium. In previous work [8, 33] it was demonstrated that an adequate oxygen concentration in the medium was essential for enzyme production by different mesophilic microorganisms. Previous studies established that caffeine degradation in *Pseudomonas* sp. is an oxygen-dependent phenomenon [27, 34]. Hence it was necessary to determine the optimum aeration conditions for production of caffeine demethylases in a bioreactor.

Caffeine demethylase production by *Pseudomonas* sp. was studied by varying the aeration rate from 0.27 to 1 vvm with agitation at 700 rpm and pH 7.0 for all the experiments. Maximum cell dry weight of 0.86 g l^{-1} was achieved in 18 h at 0.27 vvm (Figs. 2a, 3a). However, oxygen consumption was more rapid at 0.27 vvm than at other aeration rates and was slowest at 1 vvm (Figs. 2b, 3b). This indicates that *Pseudomonas* sp. is sensitive to higher aeration rates. Previous studies with *T. thermophilus* have shown that inability to adjust to high aeration rates coupled with high agitation rates resulted in an extended lag phase or slower growth [13], as seen in this study. This also explains the faster degradation of caffeine at 0.27 vvm, when complete degradation of caffeine was achieved in 18 h, than at higher aeration rates of 0.4, 0.5, and 1 vvm (Figs. 2c, 3c) indicating that higher aeration rates are detrimental to caffeine degradation by *Pseudomonas* sp. Similar results were obtained in reactor production of hemicellulose, in which higher aeration rates resulted in a decrease in enzyme production [31].

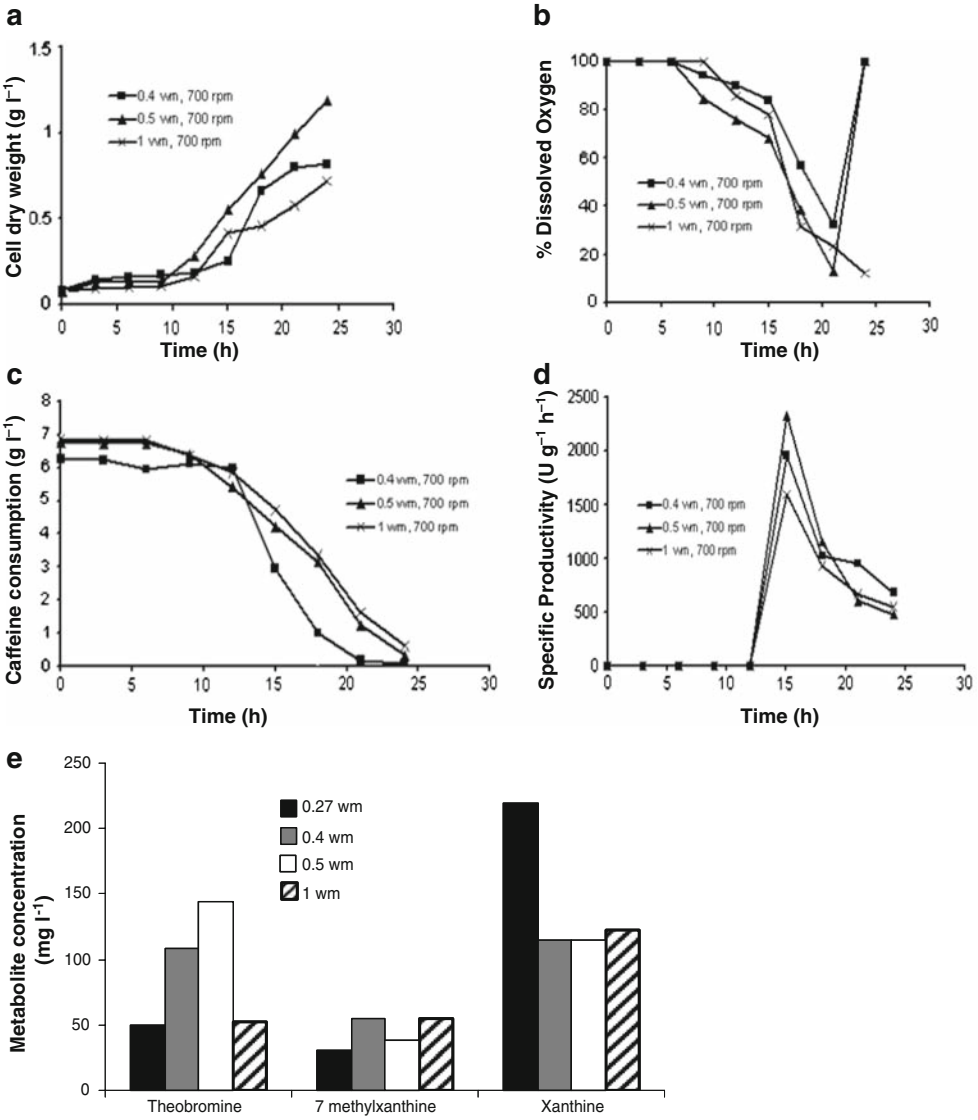

Fig. 3 Effect of aeration on **a** cell growth, **b** % dissolved oxygen, **c** caffeine consumption, **d** specific enzyme productivity, and **e** formation of metabolites in a bioreactor by *Pseudomonas* sp at 15 h of growth. Batch reactor studies were carried out at an agitation rate of 700 rpm and pH 7.0 at 30°C in all the experiments. Analysis of caffeine and metabolites was performed by RP-HPLC using a C-18 column and water–methanol 70:30 (v/v) as mobile phase

Maximum enzyme productivity (2,314 U g cell dry weight^{-1} h^{-1}) was noticed at 0.27 vvm at 18 h of growth (Figs. 2d, 3d) indicating that high aeration rates also affect caffeine demethylase production significantly. At various aeration rates, production of metabolites of the caffeine degradation pathway were analyzed and it was observed that formation of theobromine was highest at 0.5 vvm (144 mg l^{-1}) and least at 0.27 vvm, in contrast with formation of xanthine, which was highest at 0.27 vvm. The amounts of 7-methylxanthine formed at 0.4, 0.5, and 1 vvm were almost identical, and similar results were obtained for xanthine also (Fig. 3e). Overall, the amount of xanthine formed was significantly more than amounts of the other metabolites.

K_La values under different operating conditions are listed in Table 1. The values increase with increasing agitation rate. The decline in dissolved oxygen content was

Table 1 K_La values at different aeration and agitation rates

Aeration (vvm)	Agitation (rpm)	K_La (h^{-1})
0.27	400	32.7
0.27	500	47.4
0.27	700	74.2
0.27	800	73.4
0.4	700	74.4
0.5	700	74.5
1	700	75.2

also rapid under these conditions resulting in increasing K_La values. Similar results were obtained for benzaldehyde lyase production by *E. coli*, when enhancement of K_La was because of increased oxygen consumption by actively growing bacterial cells, as indicated by rapidly declining DO levels [6]. K_La values remained fairly constant (73.4–75.4 h^{-1}) when aeration rates are varied at 700 rpm. This indicated that for a given aeration rate, agitation had a pronounced and consistent effect on oxygen uptake rate, so agitation was a highly effective means of increasing the oxygen-transfer coefficient, in agreement with a previous study [7, 13]. Under conditions of maximum enzyme production (pH 7.0, agitation 700 rpm, and aeration 0.27 vvm) K_La measured by the gassing-out technique was 74.2 h^{-1}. This suggests that caffeine demethylase production was favored at high values of K_La, which is in agreement with a report for *P. putida* [27], in which it was mentioned that 3 mol oxygen are required for demethylation reaction, 1/2 mol for the urate oxidase reaction, and 2 mol for electron transport; 5.5 mol are therefore required to completely degrade caffeine to urea and carbon dioxide.

The intermediates (dimethylxanthines, for example theobromine and monomethylxanthine) of caffeine degradation are milder than caffeine and are often substituted in pharmaceutical preparations [21]. Theobromine and monomethylxanthine are synthesized by complicated chemical synthesis [15] which implies high cost. It was observed that accumulation of intermediates of the caffeine-degradation pathway largely depended on the aeration and agitation rates in the reactor. Hence recovery of the desired methylxanthine may be possible by manipulation of the reactor conditions. This is the first report on the high-level production of caffeine demethylase in bioreactors and the strain looks promising for biodecaffeination.

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