Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 2 (4): 327-336, 2007
ISSN 1816-4935

The Effect of Glucose on Growth and Degradation of Caffeine by *Pseudomonas* sp.

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Abstract: The effect of glucose on caffeine degradation by *Pseudomonas* sp., which was previously isolated in our laboratory, was investigated. Caffeine degradation was inhibited when glucose was present in free form in the medium and at a concentration > 1 g L^{-1}. Two-stage culture experiments indicated that glucose probably interferes with the induction of the enzymes involved in the caffeine degradation pathway. The addition of glucose at start and early stages of growth resulted in complete inhibition of caffeine degradation whereas addition at later stages of growth has no inhibitory effect further confirming the inhibitory effect of glucose is due suppression of caffeine catabolising enzyme, which is suggestive of catabolite repression. These findings are novel and have not been reported in other *Pseudomonas peptide* strains degrading caffeine.

Key words: Caffeine degradation, *Pseudomonas*, glucose, inhibition, two-stage culture, catabolite repression

INTRODUCTION

Microbial and enzymatic methods of caffeine degradation offer a better alternative to the present existing conventional techniques of decaffeination (Gokulakrishnan *et al*., 2005) which is one of the important steps in coffee processing keeping in view the adverse effects of caffeine on health (Cooper *et al*., 1992; James, 2004) and environment (Bressani, 1987). However, the major constraint in development of biodecaffeination techniques is the lack of a microbial strain capable of degrading higher concentrations of caffeine at an appreciable rate. In this context, a caffeine degrading strain of *Pseudomonas* capable of utilizing caffeine as the sole source of carbon and nitrogen has been isolated in our laboratory from the soil of coffee plantation area (Gokulakrishnan *et al*., 2006), which closely resembles *Pseudomonas peptide* as per 16S rRNA analysis (Dash and Gummadi, 2006a). Kinetic studies on the strain showed that the isolate could tolerate high concentration of caffeine (~12.5-15 g L^{-1} caffeine) and the minimum inhibitory concentration of caffeine was 20 g L^{-1} (Gokulakrishnan and Gummadi, 2006). This makes the strain an excellent candidate for caffeine biodegradation studies.

In preliminary studies, the strain exhibited unique caffeine metabolism when different carbon sources were supplied (Gokulakrishnan *et al*., 2006). Disaccharides (sucrose and lactose) enhanced the rate of caffeine degradation by this strain without being utilized as carbon source, whereas glucose completely inhibited the growth and caffeine degradation by this strain. On the other hand, monosaccharides like fructose and galactose were used as carbon sources. Although there are reports on the utilization of carbon sources by caffeine degrading *Pseudomonas peptide* (Blecher and Lingens, 1977), the phenomena of inhibition of caffeine degradation by glucose has not been reported

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in any other *Pseudomonas peptide* strains and appears unique to the strain used in this study. This will prove crucial in developing conditions for degradation of caffeine by the strain when natural sources of coffee and tea wastes, which contain different carbohydrates, are used. In this study an attempt has been made to delve more into the matter and to elucidate the possible mechanism of this inhibitory phenomenon of glucose on caffeine degradation by *Pseudomonas* sp.

MATERIALS AND METHODS

Microorganism

Pseudomonas sp. previously isolated was maintained on CAS agar medium which had the following composition (g L^{-1}): Na_2HPO_4, 0.12; KH_2PO_4, 1.3; $CaCl_2$, 0.3; $MgSO_4$. 7 H_2O, 0.3.; caffeine, 1.2; sucrose, 5 and agar, 25. The strain was sub-cultured every alternate day to maintain caffeine degrading ability.

Chemicals

Caffeine (>99% purity) was obtained from Merck, India. Theobromine, theophylline, 7-methylxanthine and xanthine were obtained from Sigma. All other chemicals were procured from Hi-Media, India.

Media

Nutrient Broth used for seed culture had the following composition (g L^{-1}): beef extract 1; yeast extract 2; peptone, 5; NaCl, 5. The composition of basal medium (BM) was as follows (g L^{-1}): Na_2HPO_4, 0.12; KH_2PO_4, 1.3; $CaCl_2$, 0.3; $MgSO_4$. 7 H_2O, 0.3. CAS medium consisted of BM supplemented with 5 g L^{-1} sucrose and 1.2 g L^{-1} caffeine. Carbon sources added to the medium were sterilized separately and then mixed with the medium under aseptic conditions. The initial concentration of glucose and sucrose in the medium was 5 g L^{-1}. Stock solution of 625 g L^{-1} glucose was prepared and autoclaved separately for intermittent additions. For an alternate nitrogen source caffeine was replaced by 2.2 g L^{-1} ammonium sulphate in CAS medium as per experimental requirement. The initial pH of the medium was adjusted to 6.0 before autoclaving.

Flask Culture Experiments

For flask culture experiments, three loopfuls of culture from CAS agar plate cultured at 30°C for 36 h was transferred to 25 mL sterile Nutrient Broth and incubated in a rotary shaker (Orbitex, Scigenics Biotech, India) at 30°C and at 180 rpm. After the seed OD_{600} reached ~1.3, 6% (v/v) of the culture was transferred to 25 mL of CAS medium in 100 mL Erlenmeyer flasks and incubated on a rotary shaker at 180 rpm and at 30°C. At regular intervals, samples were collected and analyzed for cell growth, caffeine degradation and sugar utilization. Three sets of experiments were performed as described below:

Effect of Addition of Glucose with Other Sugars and at Different Concentrations

The medium for this experiment consisted of Basal Medium (BM) supplemented with glucose and other sugars at 5 g L^{-1} concentration, separately or in combination. When two sugars were used, composition of both the sugars was adjusted such that the total carbon content was 5 g L^{-1}. The carbon sources were autoclaved separately and added just before inoculation. 6% (v/v) of the seed culture at OD_{600} ~1.3 was transferred to 25 mL of medium in 100 mL Erlenmeyer flask and incubated on a rotary shaker at 180 rpm and at 30°C. Samples were collected at regular intervals and analyzed for cell growth, caffeine degradation and sugar utilization. All experiments were performed in triplicates under identical conditions and all results had a standard deviation of ±1 to ±3% about the mean.

Two Stage Culture Experiments

Two stage culture experiments were performed in order to determine the mechanism of inhibition of caffeine degradation by glucose. Cells were initially grown in glucose-ammonium sulphate medium and CAS medium and were harvested by centrifugation at 10000 x g (Centrifuge 5810 R, Eppendorf, Germany) at the mid log phase (~ 10 h), followed by twice washing with 50 mM potassium phosphate buffer (pH 7.0). Equal amount of cells (0.016 g L^{-1} dry weight) were transferred in the second stage in the following manner: (i) from glucose-ammonium sulphate medium to CAS medium, (ii) from CAS medium to glucose-caffeine medium and (iii) from CAS medium to fresh CAS medium as control. Caffeine degradation and sugar consumption were monitored at different time intervals. In all second stage experiments, 0.016 g dry cell weight L^{-1} of medium was maintained. Experiments were performed in triplicates and all results had a standard deviation of ±1 to ±5% about the mean.

Intermittent Addition of Glucose

In this set of experiments, 6% (v/v) of the seed culture at OD_{600} ~1.3 was transferred to 25 mL of CAS medium in 100 mL Erlenmeyer flasks and incubated on a rotary shaker at 180 rpm and at 30°C. One milliliter of sterile glucose solution (625 g L^{-1}) was added to the culture at 0, 2, 3, 5, 7 and 8 h of growth. The control consisted of CAS medium without addition of glucose. Samples were collected at the above mentioned time points and analyzed for cell growth, caffeine degradation and sugar utilization. Experimental values reported are an average of three sets with ±1% to ±5% standard deviation about the mean.

Analytical Determinations

Cell density in the medium was monitored by measuring the optical density at 600 nm in a spectrophotometer (Biorad, India) (OD_{600} of 0.5 corresponds to 0.379 g dry weight L^{-1}). Caffeine, theobromine, 7-methylxanthine and xanthine were estimated by HPLC (Agilent 1100 series) equipment using a ZORBAX C-18 column with 10 mM ammonium phosphate buffer (pH 2.5)/acetonitrile (4:1, v/v) as mobile phase at a flow rate of 1 mL min^{-1} and at 28.5°C. Detection of caffeine was done at 254 nm. The concentrations of carbon source in the medium were estimated by 3, 5-dinitrosalicylic acid method (Miller, 1959). Sucrose and lactose were hydrolyzed with 3N HCl by heating at 100°C for 15 min and the reducing sugars released were analyzed by 3, 5-dinitrosalicylic acid method.

RESULTS AND DISCUSSION

Bacterial strains belonging to the genus *Pseudomonas* are well known for their capability to metabolize compounds that are toxic to other microbes and this attribute has evolved as an adaptation to substrate availability in the natural environment. However catabolite repression is often associated with the degradation of such compounds whereby the catabolism of the xenobiotic is suppressed in the presence of simple sugars like glucose (Collier *et al*., 1996). Preliminary studies pertaining to caffeine degradation have shown that carbon sources have a distinctive effect on the growth and caffeine degrading ability of *Pseudomonas* sp. used in this study (Gokulakrishnan *et al*., 2006). Particularly, the inhibitory effect of glucose on caffeine degradation and growth of this strain has not been reported for any caffeine degrading bacterial strains so far, which suggests the unique metabolic attributes that this strain might have in relation to caffeine catabolism and carbon source utilization. Results obtained indicate the possible role of glucose in suppression of caffeine degrading enzymes which account for its inhibitory effect.

Effect of Addition of Glucose with Other Sugars

In the previous reported study (Gokulakrishnan *et al*., 2006), it was observed that *Pseudomonas* sp. could effectively utilize monosaccharides like fructose and galactose as carbon

sources along with caffeine. Glucose inhibited the degradation of caffeine whereas glucose containing disaccharides like sucrose and lactose did not; rather the rate of caffeine degradation was enhanced in their presence. It was therefore necessary to investigate whether the inhibitory effect of glucose holds good when glucose is present in the free form or it is masked when glucose is present along with any other monosaccharide that the strain is capable of utilizing. Also, it was necessary to determine the concentration of glucose necessary to cause inhibition. For this purpose, the degradation of caffeine was studied in medium containing glucose with other monosaccharides like fructose and galactose (where glucose was present in the free form) and in presence of disaccharides like sucrose and lactose (where glucose was present as a one of the monomers of the disaccharides). Caffeine degradation by *Pseudomonas* sp. was also studied by varying the initial concentration of glucose in the medium from 0.05 to 5 g L^{-1}.

Results showed that complete inhibition of caffeine degradation was observed when glucose was present in free form along with another sugar. However, this inhibition effect was not seen when glucose was present as a monomer in the disaccharide (sucrose or lactose) (Fig. 1a). The effect of glucose concentration on caffeine degradation was studied by varying the initial concentration of glucose in the medium. Caffeine degradation was completely inhibited when the initial concentration of glucose was 5 g L^{-1} (Fig. 1b) and in the range 1-5 g L^{-1} of sucrose (data not shown). The inhibition effect decreased when the medium contained <0.5 g L^{-1} glucose (Fig. 1b). However, degradation in the presence of all concentrations of glucose was lower than control where the isolate was grown in the presence of sucrose.

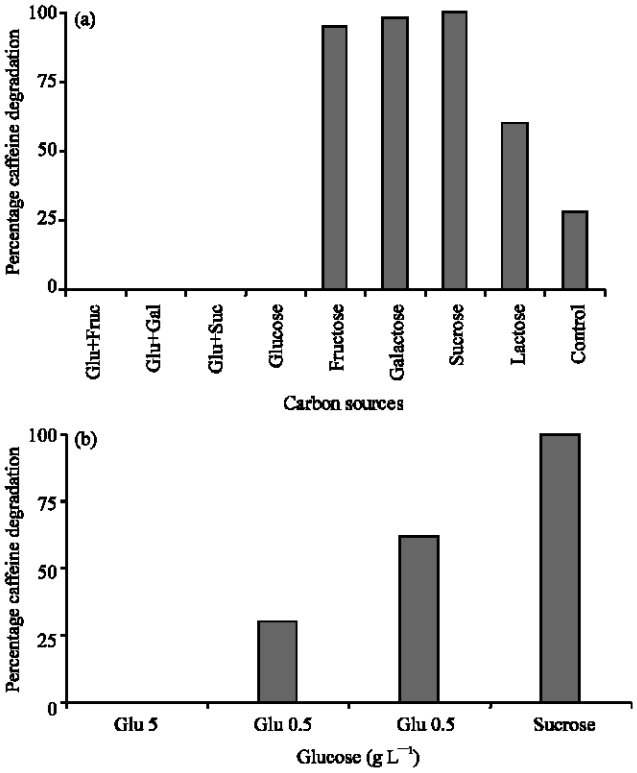

Fig. 1: The inhibitory effect of glucose on the caffeine degradation by *Pseudomonas* sp. (a) effect of different sugars and in combination with glucose on caffeine degradation. (b) effect of glucose concentration on caffeine degradation. All experiments were performed in triplicates under identical conditions and all results had a standard deviation of ±1 to ±3% about the mean

These results are in contrast to the findings of Asano *et. al.* where they reported that glucose enhanced the degradation of caffeine to theobromine by *Pseudomonas peptide* (Asano *et al*., 1993). The ability of the strain to utilize galactose but not lactose is also in contrast to the caffeine degrading *Pseudomonas* strain isolated by Bletcher and Lingens (Blecher and Lingens, 1977) that was capable of utilizing glucose, fructose and lactose but not galactose as carbon source. These differences make the strain unique from other caffeine degrading strains isolated so far and probably it has a different pathway in metabolizing carbohydrates, which is not surprising since hexoses, in *Pseudomonas* sp. can be catabolized in more than one route (Vicente and Canovas, 1973). Therefore, glucose in the free form or some intermediate of glucose catabolism unique to this strain is possibly responsible for this inhibition. Also from the results it is clear that since disaccharides were not utilized by the strain, the strain lacks the enzymes needed for the conversion of disaccharides to free glucose and therefore no inhibition of caffeine degradation was observed in presence of disaccharides.

It was also noted that the inhibitory effect of glucose was concentration dependent since caffeine degradation was completely inhibited when the initial concentration of glucose in the medium was >1 g L^{-1} and decreased when the medium contained <0.5 g L^{-1} glucose. Similar effect was seen in case of 2-chlorphenol degradation by a strain of *Pseudomonas peptide* (Fakhruddin and Quilty, 2005) which further supports study.

Effect of Glucose on Induction of Caffeine Degrading Enzymes

One of the possible reasons for the inhibitory effect of glucose is the repression of synthesis of the enzymes involved in caffeine degradation. The inducible nature of caffeine degrading enzymes has been established from earlier reports (Woolfolk, 1975; Ogunseitan, 2002) and also from studies carried out on the strain used in the present study (Gummadi and Santosh, 2006). Since these enzymes are inducible in nature, the presence of glucose might suppress their induction due to which caffeine degradation is inhibited as an outcome of catabolite repression. This hypothesis is strengthened by the earlier findings where glucose has been shown to repress the induction of enzymes responsible for the degradation of various compounds like catechlol and chlorcatechol, methyl phenol and benzyl alcohol (Holtel *et al*., 1994; Muller *et al*., 1996; McFall *et al*., 1997).

This hypothesis was tested by two stage culture experiments, an approach that has been used in previous studies to establish enzyme induction (Naidu *et al*., 2001). According to this strategy, cells were initially grown in a medium containing glucose and ammonium sulphate and then transferred to CAS medium at mid log phase of growth. Since caffeine was absent in the first stage of growth, the cells were assumed not to be induced with caffeine degrading enzymes. The control consisted of an equal amount of cells, initially grown in CAS medium for 10 h being transferred to a new CAS medium. Equal amount of cells from CAS medium were also transferred to a medium containing glucose and caffeine, presuming that the cells were induced at the time of transfer to second stage.

The cells transferred from CAS medium to glucose and caffeine medium showed significant growth and caffeine degradation despite the presence of both caffeine and glucose in the medium (Fig. 2a and b). The glucose utilization in the second stage is ~90% (Fig. 2c). It was observed that CAS grown cells when transferred to glucose medium exhibited 90% caffeine degradation in 12 h. Since the cells had been grown in CAS medium during first stage in the absence of glucose, the caffeine degrading enzymes are already present when the cells are transferred to the second stage (caffeine and glucose medium). On the other hand, the cells transferred from glucose-ammonium sulphate medium to CAS medium showed little growth in CAS medium and showed very less caffeine degradation (<5% in 18 h) (Fig. 2a and b). This can be attributed to the fact that glucose present in the first stage of growth (the glucose-ammonium sulphate medium) inhibited the induction and expression of caffeine degrading enzymes.

The two stage culture experiment supports the hypothesis that inhibition of caffeine degradation by glucose is due to initial inhibition of the expression of the caffeine degrading enzymes and that the

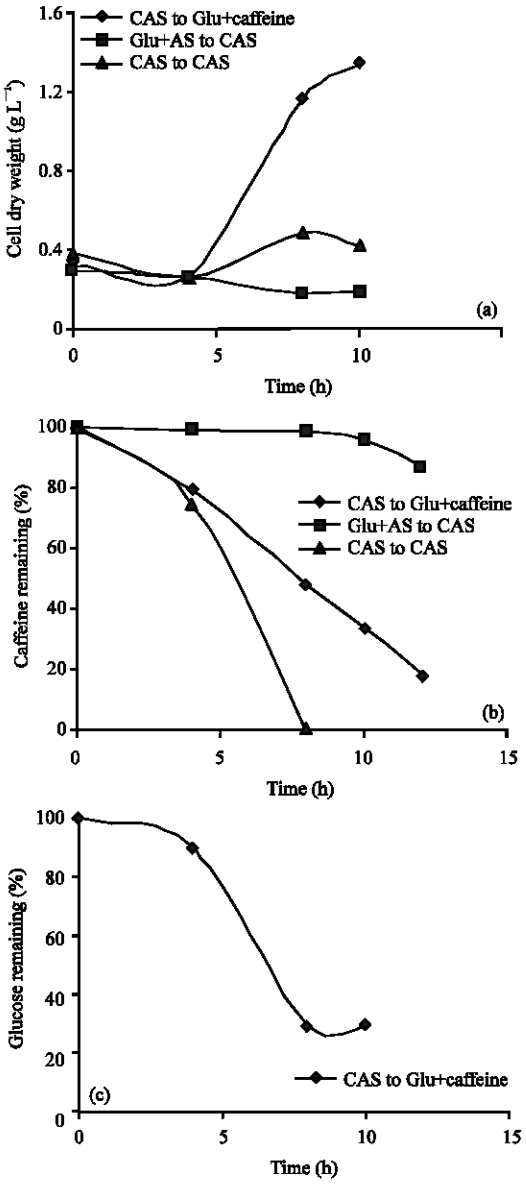

Fig. 2: Two stage culture experiment to show the effect of glucose on induction of caffeine degrading enzymes by *Pseudomonas* sp. Equal amount of cells initially grown in glucose-ammonium sulphate medium and CAS medium were transferred to CAS and glucose-ammonium sulphate, respectively. Control consisted of cells transferred from CAS medium to fresh CAS medium. (a) cell growth (b) caffeine degradation profile and (c) glucose consumption profile in the second stage culture. All experiments were performed in triplicates under identical conditions and all results had a standard deviation of ±1 to ±5% about the mean

phenomena of catabolite repression occurs in this strain also. Degradation of caffeine in *Pseudomonas* follows sequential demethylation leading to formation of theobromine, 7-methylxanthin and xanthine by caffeine N-demethylases (Dash and Gummadi, 2006b) and it also happens so in the strain used in

this study (Dash and Gummadi, 2006a). Glucose has been shown to repress the gene coding for a demethylase in case of the fungus *Nectria haematococca* in an earlier report (Khan and Straney, 1999). Furthermore, along with glucose, intermediates of glucose metabolism like gluconic acid have been reported to cause the repression of the enzymes involved in catechol degradation at transcriptional level (McFall *et al*., 1997). Probably, such a mechanism also operates in *Pseudomonas* sp. used in this study in relation to the metabolism of caffeine.

Effect of Addition of Glucose at Various Time Points of Growth

From the above experiment, it was established that inhibitory effect of glucose on caffeine degradation is due to inhibition of enzyme expression. Therefore further experiments were needed to find out whether this catabolite repression is effective only at the initial stages of growth of the bacteria in caffeine medium or does it occur at all stages. For this purpose, glucose was added to CAS grown culture at various time points such that final concentration of glucose was 5 g L^{-1}.

When glucose was added at early time points (0 and 2 h), there was no significant difference in cell growth compared to the control (CAS medium without addition of glucose) (Fig. 3a). Also, complete inhibition of caffeine degradation was observed when glucose was added at 0 h, whereas very little degradation (~5-8%) was observed when glucose was added at 2 h (Fig. 3b). The glucose utilization was also observed to be negligible at the early addition (Fig. 3c). This indicates clearly that the bacterium is not able to metabolize caffeine when glucose is added during initial stages of growth and neither is utilized since there is no growth of the strain.

The situation is however different when glucose was added at the time of active growth of the strain in caffeine medium. Significant growth was observed when glucose was added at later points (3 to 8 h) (Fig. 3a). Clearly, the possibility of diauxic growth is ruled out as no growth was noticed when glucose was present in the initial stages along with caffeine. Supplementing this, significant increase in degradation was observed when glucose was added at the later stages and was observed to be was > 90% when glucose was added at 8 h (Fig. 3b). Moreover, glucose was also metabolized by the strain when added at later stages and at 8h of glucose addition, glucose consumption was around 100% (Fig. 3c). A maximum dry cell weight of 2.3 g L^{-1} was obtained when glucose was added at 8 h with a yield of 0.46 g cell dry weight g^{-1} glucose suggesting that glucose was being used as an additional carbon source. It has also been observed that sucrose was not consumed (data not shown).

This proves that the inhibitory effect of glucose on caffeine degradation was effective only when glucose was present in the initial stages of growth whereas degradation was enhanced when glucose was added at later stages of growth (Fig. 3). It further establishes that the addition of glucose at early stages of growth inhibits the induction and expression of caffeine degrading enzymes that are synthesized in the lag phase. This effect ceases to exist when glucose was added at later stages of growth because already the genes necessary for expression of caffeine degrading enzymes would have been transcribed in the cell and the strain is active growing in caffeine medium. Once sufficient biomass has been achieved, *Pseudomonas* sp. used in this study is also able to utilize glucose as carbon source.

The next step was to investigate whether this phenomenon is limited to caffeine or does it extend to the other methylxanthines, which are metabolites of caffeine degradation pathway. To check this, the isolate was grown in CAS medium in which caffeine was replaced by theobromine, theophylline, 7-methylxanthine and xanthine (methylxanthines at 1 g L^{-1} concentration). It was observed that isolate could metabolize theobromine, 7-methylxanthine and xanthine but not theophylline. Similar experiment was performed in the presence of glucose and found that isolate could not grow and degrade caffeine metabolites. Theophylline was not utilized in both the cases because it is not a metabolite in the degradation pathway of the bacteria, as shown earlier (Dash and Gummadi, 2006a). It indicates that enzymes bringing the conversion of caffeine to its metabolites are repressed by glucose. More study on the molecular aspects of regulation is required to arrive at a decisive theory.

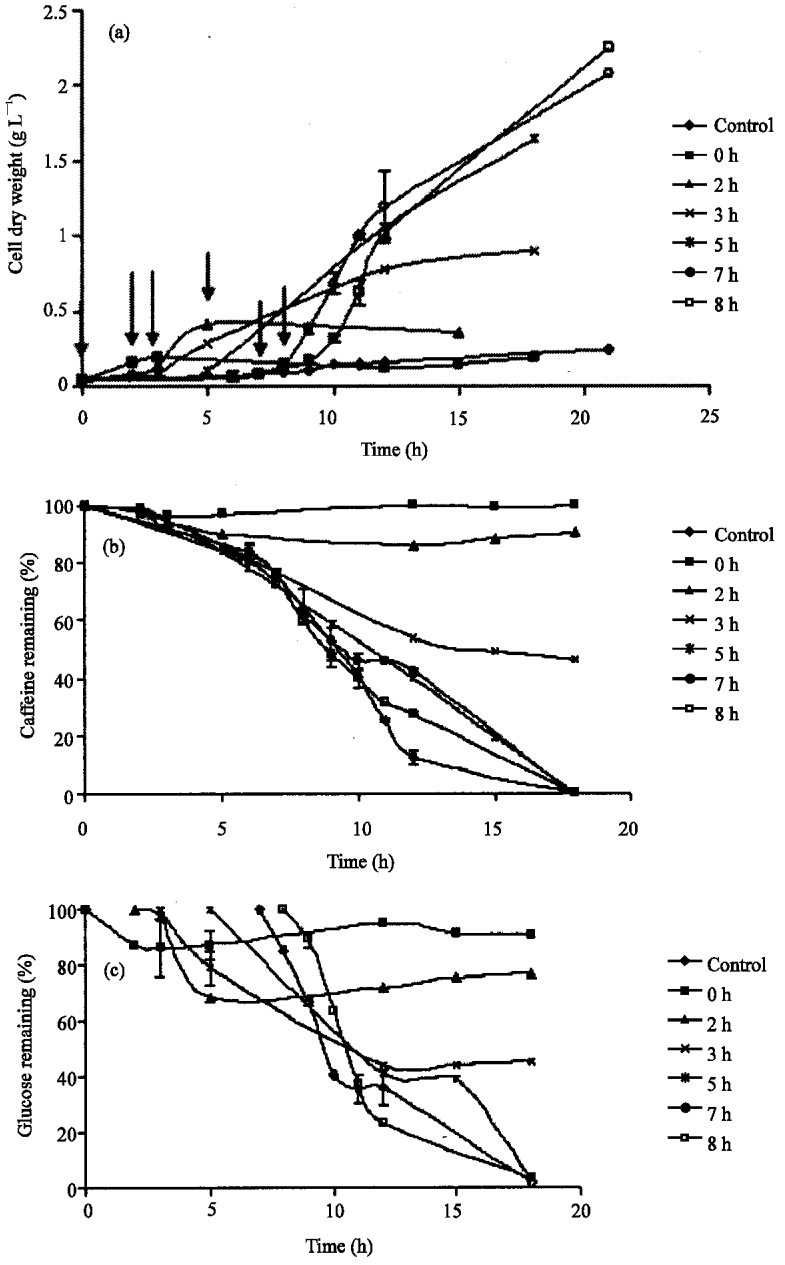

Fig. 3: Effect of addition of glucose at different growth points on caffeine degradation by *Pseudomonas* sp. Glucose was added from stock solution to achieve a concentration of 5 g L^{-1} in the medium at 0, 2, 3, 5, 7 and 8 h, respectively. Control consisted of growth of cells in CAS medium. (a) cell growth, (b) caffeine degradation and (c) sugar consumption were monitored at regular intervals. Data presented is the average of triplicates with ±1 to ±5% standard deviation about the mean

These findings throw light on the complex interaction between glucose and caffeine metabolism. Either the pathways of metabolism of both these compounds are interlinked or glucose has effect on the regulation of caffeine degrading enzymes. *Pseudomonas* sp. proves to be an attractive model to understand the degradation of caffeine on the molecular level, which in turn will prove beneficial in developing biological decaffeination techniques.

ACKNOWLEDGMENT

We are grateful to Department of Science and Technology, India for their financial support.

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