Activity and stability of caffeine demethylases found in *Pseudomonas putida* IF-3.

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Abstract

In order to establish the viability of using a biological system to decaffeinate coffee, a bacterium (*Pseudomonas putida* IF-3) capable of living on caffeine as its only source of carbon and nitrogen was studied. Resting cell suspensions and cell-free extracts of *P. putida* IF-3 were tested to assess their ability to degrade caffeine, and to determine their capacity to retain activity at different temperatures. A method to quantify cell lysis was developed; this method allowed comparison of cell free extract and resting cell caffeine degradation rates. Caffeine degradation rates for cell free extracts were found to be 55 times faster than previously reported *P. putida* data. Resting cells degraded caffeine 12 times faster than cell free extracts, at 22°C. However, both systems were equivalently active at 50°C. Finally, resting cells were found to be more stable than cell free extracts; this was significantly more evident at 50°C than at 22°C.

Résumé

Une bactérie (*Pseudomonas putida* IF-3), capable d'utiliser la caféine comme seule source de carbone et d'azote, a été étudiée dans le but d'établir la viabilité de l'utilisation d'un système biologique pour la décaféinisation du café. Des suspensions de cellules à l'état dormant ainsi que des extraits bactériens (exempts de cellules) ont été évalués pour déterminer leur habilité à dégrader la caféine et leur capacité à maintenir leur activité à différentes températures. Une méthode pour quantifier la rupture de cellules a été développée : cette méthode a permis de comparer les taux de dégradation de caféine par les cellules dormantes avec celui des extraits bactériens. Les taux de dégradation de caféine par les extraits bactériens se sont révélés être 55 fois plus élevés que les taux rapportés antérieurement pour des extraits de *P. putida*. À 22°C, les taux de dégradation de caféine par les cellules dormantes se sont révélés être 12 fois plus élevés que les taux de dégradation par les extraits bactériens. Cependant, à 50°C, on a constaté que les deux systèmes avaient des activités très similaires. Finalement, il a été établi que les cellules dormantes étaient plus stables que les extraits bactériens et que ce phénomène était plus évident à 50°C qu'à 22°C.

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Nomenclature

Units are as below, unless otherwise specified in the text.

A	Absorbance
$A_{340\ nm}$	Absorbance measured at 340 nm
BSA	Bovine serum albumin
cCFE	Centrifuged cell free extract
CFE	Cell free extract
EDTA	Ethylenediaminetetraacetic acid
g cells	grams of cells (g)
g protein	grams of protein (g)
HPLC	High performance liquid chromatography
k	Lysis rate constant (min^{-1})
M	Molar (mol / L)
mM	Millimolar (mmol / L)
N	Normal (mol of proton equivalent / L)
NAD	Nicotinamide adenine dinucleotide
NAD^+	Nicotinamide adenine dinucleotide, oxidized form
NADH	Nicotinamide adenine dinucleotide, reduced form
PAGE	Polyacrylamide gel electrophoresis
RC	Resting cells
SDS	Sodium dodecyl sulfate
t	time (min)
TEMED	N,N,N',N'-tetramethylethylenediamine
Λ	Activity (mmol caffeine $\cdot$ g $cells^{-1}$ $\cdot$ min^{-1})
η	Lysis efficiency

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Introduction

The world's coffee market is estimated at 70 billion dollars per annum (OSORIO 2003), and decaffeinated coffee accounts for more than 12 % of the coffee market in the United States (CASTLE 1994). Commercial decaffeination methods use solvents to remove caffeine from whole, green coffee beans, prior to roasting. Extraction agents include methyl chloride, ethylene acetate, supercritical CO_2, and hot water.

Up to now, a process that targets freshly brewed coffee for decaffeination has not been developed. Such a process could turn decaffeination into a domestic operation, eliminating the requirement for industrial facilities dedicated to decaffeination, and eliminating the consumer's need to buy decaffeinated coffee. A decaffeination method that targets hot beverages should be highly selective for caffeine, in order to reduce caffeine content without affecting flavor.

The use of a bacterium, *Pseudomonas putida,* for biological decaffeination of beverages has been considered in the past (HAAS and STIEGLITZ 1980b; MIDDLEHOVEN 1982; HAAS and STIEGLITZ 1980a). Gaulin (GAULIN 2003) proved that selective caffeine removal by *P. putida* was possible in aqueous solutions. Cell free extracts from *P. putida* having caffeine degrading activity have been reported by Asano (ASANO et al. 1994), Hohnloser (HOHNLOSER et al. 1980), and Sideso (SIDESO et al. 2001). However, none of the published studies showed the effect of temperature on the caffeine degradation rates by *P. putida* or *P. putida*'s cell free extracts. Temperature is a key

factor if a biological method to decaffeinate hot beverages is to be developed. This thesis investigates the effect of temperature on caffeine degradation rates by resting cells and cell free extracts of *P. putida* IF-3 in aqueous solutions.

Objectives

- To quantify the activity of caffeine demethylases in resting cells and cell free extracts of *Pseudomonas putida* IF-3 at different temperatures.

- To determine the stability of caffeine demethylases in resting cells and cell free extracts of *Pseudomonas putida* IF-3 at different temperatures.

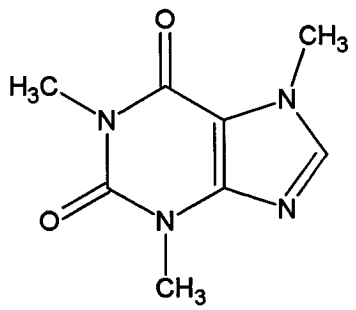
Hypotheses

- When compared on the same basis, resting cells of *Pseudomonas putida* IF-3 degrade caffeine in buffered aqueous solutions at a faster rate than its cell free extract.

- Temperatures near coffee-drinking temperature will cause a rapid reduction in the caffeine degradation rates of resting cells, and of cells free extracts of *Pseudomonas putida* IF-3 in buffered, aqueous solutions.

1. Literature Review

1.1 Caffeine



Caffeine
(1, 3, 7-Trimethylxanthine)

Figure 1-1: Caffeine Molecule

Caffeine (Figure 1-1) is a white, odorless, powder with a slightly bitter taste. It is soluble in water at about 20 g / L at 20°C, 150 g / L at 80°C, and 650 g / L at 100°C (WORLD HEALTH ORGANIZATION: INTERNATIONAL AGENCY FOR RESEARCH ON CANCER 1991). Caffeine has very little ability to complex with metal ions in contrast with that of EDTA (KOLAYLI et al. 2004). EDTA is the most widely used chelator in analytical chemistry due to its ability to form strong complexes with most metal ions (HARRIS 1999). Caffeine is stable except in strong solutions of caustic alkalis (WORLD HEALTH ORGANIZATION: INTERNATIONAL AGENCY FOR RESEARCH ON CANCER 1991).

Caffeine is found in more than 60 plant species throughout the world. It occurs in dry green beans of arabica and robusta coffees at levels of 0.9-1.4 % and 1.5-2.6 %

respectively. The level of caffeine in tea (*Camellia sinensis*) is affected by a wide variety of parameters, and can be as high as 5 %. The weighted average of caffeine level in tea sold in the United States is 3.0 %. Caffeine levels in the mate plant (*Ilex paraguanensis*) vary from 0.7 % to 2 % (WORLD HEALTH ORGANIZATION: INTERNATIONAL AGENCY FOR RESEARCH ON CANCER 1991).

At least one caffeine-containing food or beverage is consumed by most adults and children per day; 70 % of the caffeine consumed is in the form of coffee, 16 % in tea, 12 % in soft drinks and 2 % in other food sources (FRARY et al. 2005). The caffeine content in brewed coffee is between 50 to 120 mg per 150 mL cup (1.5 – 4 mM) depending on the preparation method. Decaffeinated coffee contains between 0 and 6 mg of caffeine per 150 mL cup (0.2 mM). Caffeine levels in brewed tea are similar for black and green tea, ranging from 20 to 60 mg per 150 mL cup (0.7 – 2 mM) (WORLD HEALTH ORGANIZATION: INTERNATIONAL AGENCY FOR RESEARCH ON CANCER 1991).

At low doses (up to 2 μg / mL in blood), caffeine stimulates the central nervous system, and this effect is perceived by many caffeine users as beneficial. High blood concentration (10-30 μg / mL) of caffeine may produce restlessness, excitement, tremor, tinnitus, headache and insomnia. Caffeine can induce alterations in mood and sleep patterns, increase urine production and gastric juice secretion, alter myocardial function, induce hypertension and arrhythmia, and increase plasma catecholamine levels and plasma renin activity. The most important metabolic pathway for the breakdown of

caffeine in humans is demethylation of the molecule (95 %), yielding only 5 % trimethyl derivatives (WORLD HEALTH ORGANIZATION: INTERNATIONAL AGENCY FOR RESEARCH ON CANCER 1991).

1.2 Nicotinamide Adenine Dinucleotide (NAD)

In the case of caffeine demethylation, mediated by the demethylases present in cell free extracts of *Pseudomonas putida,* NADH is required for the reaction to take place (ASANO et al. 1994; HOHNLOSER et al. 1980). NADH is oxidized during caffeine demethylation, producing theobromine, formaldehyde, water and NAD^+ (Figure 1-7). The produced NAD^+ could then undergo reduction by reacting with water and formaldehyde (the reaction being catalyzed by the formaldehyde dehydrogenases present in the cell free extracts) to yield formic acid, NADH and proton (Figure 1-8).

The structure of the oxidized form of NAD is shown in Figure 1-2. It is drawn as a cation, with one positive charge on the nicotinamide group, but in fact at pH 7.5 the acidic groups of the two phosphate groups are in the ionized form, giving a net negative charge on the molecule (DIXON and WEBB 1979). At neutral pH, the oxidized form of NAD exists as NAD^- and the reduced form as $NADH^{2-}$, due to the negative charges in the ionized phosphate groups. It is customary, however, to ignore the phosphate groups and to write the charge only on the nicotinamide part of NAD (Figure 1-2). When referring to the coenzyme without specifying whether the oxidized of reduced form is meant, NAD is used (DIXON and WEBB 1979).

The active group in NAD is the nicotinamide segment (Figure 1-2), which acts by undergoing reduction or oxidation. Thus, it is customary to show explicitly only the nicotinamide part (Figure 1-3) (SOLOMONS 1996). The reduction and oxidation are

shown in Figure 1-3, where 2H represents two reducing equivalents. The transition from the oxidized to the reduced form of NAD happens by incorporation of one hydrogen atom and one electron to the nicotinamide group, thus removing the positive charge. For this reason, the oxidized form is usually written as NAD^+, and the reduced form as NADH (DIXON and WEBB 1979).

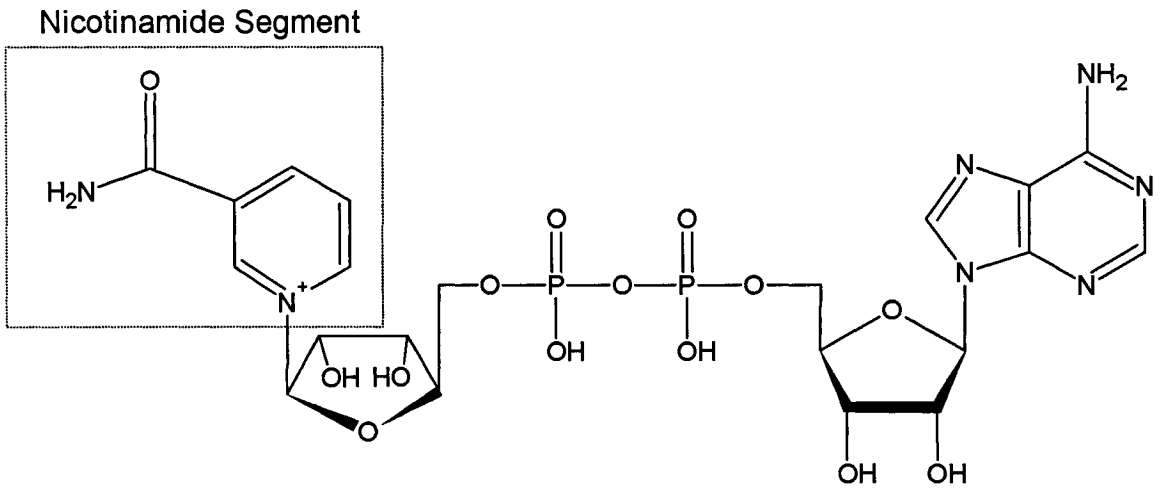


Figure 1-2: NAD, oxidized form

When reduction occurs, the pyrinidium ring in NAD^+ is converted to a nonaromatic ring in NADH. The extra stability of the pyridine ring is lost in this change, and as a result the free energy of NADH is greater than that of NAD^+ (DIXON and WEBB 1979). NAD can be reversibly reduced by one substance and oxidized by another, that is why NAD acts as a carrier of reducing equivalents from molecule to molecule (DIXON and WEBB 1979).

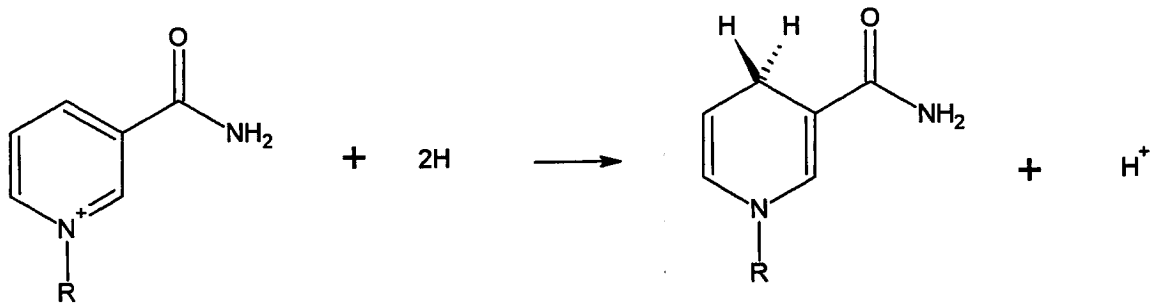


Figure 1-3: Reduction of NAD

The ultraviolet absorption spectrum varies from the oxidized to the reduced form. NAD^+ shows only a band at 260 nm, due to the purine and the pyridine rings. NADH shows an additional band at 340 nm due to the quinonoid bond structure of the reduced nicotinamide ring (DIXON and WEBB, 1979).

NADH is extremely unstable in acid, but relatively stable in alkaline solutions, while NAD^+ is fairly stable in acid and less stable in alkali. At neutral pH NAD^+ is more stable than NADH (DIXON and WEBB, 1979).

1.3 *Pseudomonas putida*

Pseudomonas putida is a rod-shaped, gram-negative bacterium. It is a strict aerobe and possesses one or more flagella (BROCK, 1979). Its size varies between 0.5 µm by 1.5 µm and 1.0 µm by 4.0 µm (Figure 1-4). It produces a characteristic water-soluble yellow-green fluorescent pigment (BROCK, 1979).

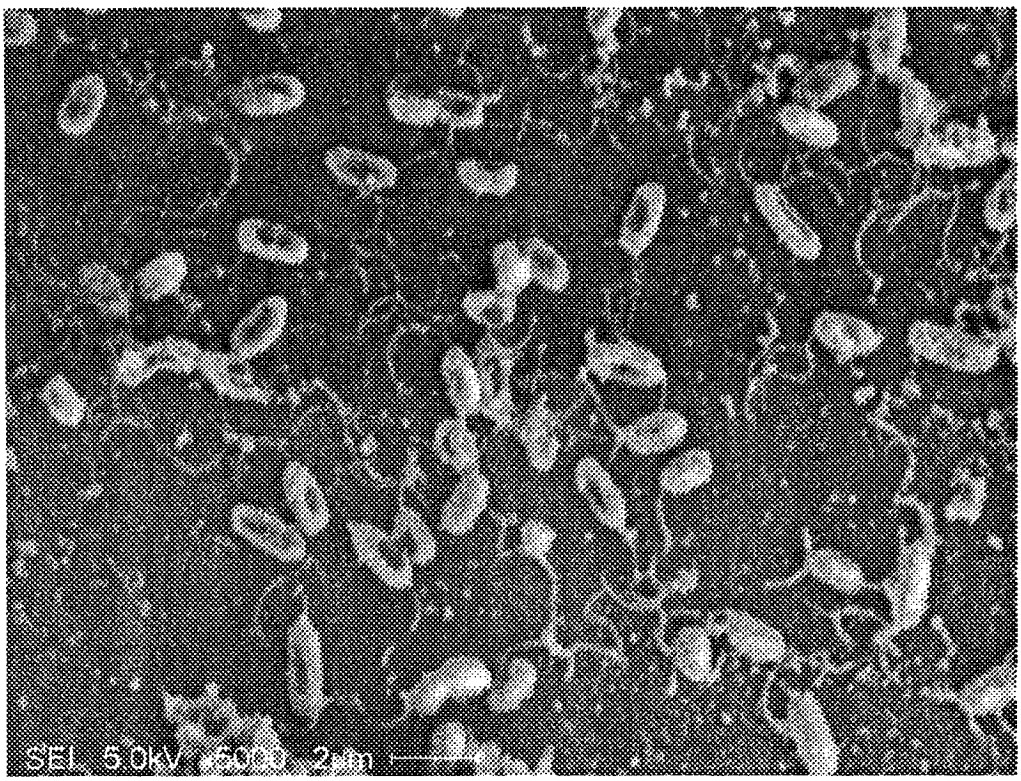


Figure 1-4: Scanning electron micrograph of *Pseudomonas putida* IF-3. Magnification, 5000×.

Despite negligible morphological and physiological differences, not all *Pseudomonas putida* species can degrade caffeine (BLECHER and LINGENS 1977). However, strains of *Pseudomonas putida* able to grow on caffeine as a sole source of carbon and nitrogen have been isolated from soil by various researchers (WOOLFOLK 1975), (BLECHER and LINGENS 1977), (MIDDELHOVEN 1982), (ASANO et al. 1993), (KOIDE et al. 1996), (YAMAOKA-YANO and MAZZAFERA 1999), and from domestic wastewater by Ogunseitan (OGUNSEITAN 1996).

Blecher (BLECHER and LINGENS 1977) proposed that *P. putida* degrades caffeine (1,3,7-trimethylxanthine) via three successive demethylation steps (Figure 1-5), followed by oxidation of xanthine to uric acid (Figure 1-5), breakage of the purine ring (Figure 1-6), and the eventual accumulation of urea in the growth medium. Glyoxilic acid, the other end product of the purine breakdown (Figure 1-5), is completely mineralized by *P. putida* (BLECHER and LINGENS 1977). Confirming evidence for the demethylation sequence was put forward by Asano (ASANO et al. 1993; ASANO et al. 1994), Koide (KOIDE et al. 1996) and Yamaoka-Yano (YAMAOKA-YANO and MAZZAFERA 1999). *N*-demethylation has been reported to be an oxidative reaction that proceeds through the formation of an *N*-hydroxymethyl intermediate. This compound is unstable and breaks down to form a demethylated product and formaldehyde (BLECHER and LINGENS 1977). Caffeine demethylases have been shown to be inducible enzymes in *P. putida* strains that are capable of degrading caffeine (BLECHER and LINGENS 1977; ASANO et al. 1993; HOHNLOSER et al. 1980; HOHNLOSER et al. 1980; OGUNSEITAN 2002). Despite being able to degrade methylated xanthines, caffeine degrading strains of *Pseudomonas putida* cannot degrade methylated uric acids or urea (BLECHER and LINGENS 1977; YAMAOKA-YANO and MAZZAFERA 1999). The xanthine oxidase in *P. putida* has a wide substrate specificity (YAMAOKA-YANO and MAZZAFERA 1999), and it can oxidize *N*-methylxanthines; however, the uricase responsible for the breakage of the purine ring in uric acid is highly specific, and cannot break down the oxidized *N*-methylxanthines (BLECHER and LINGENS 1977). (As a result methylated uric acids will accumulate in small quantities in liquid cultures.)

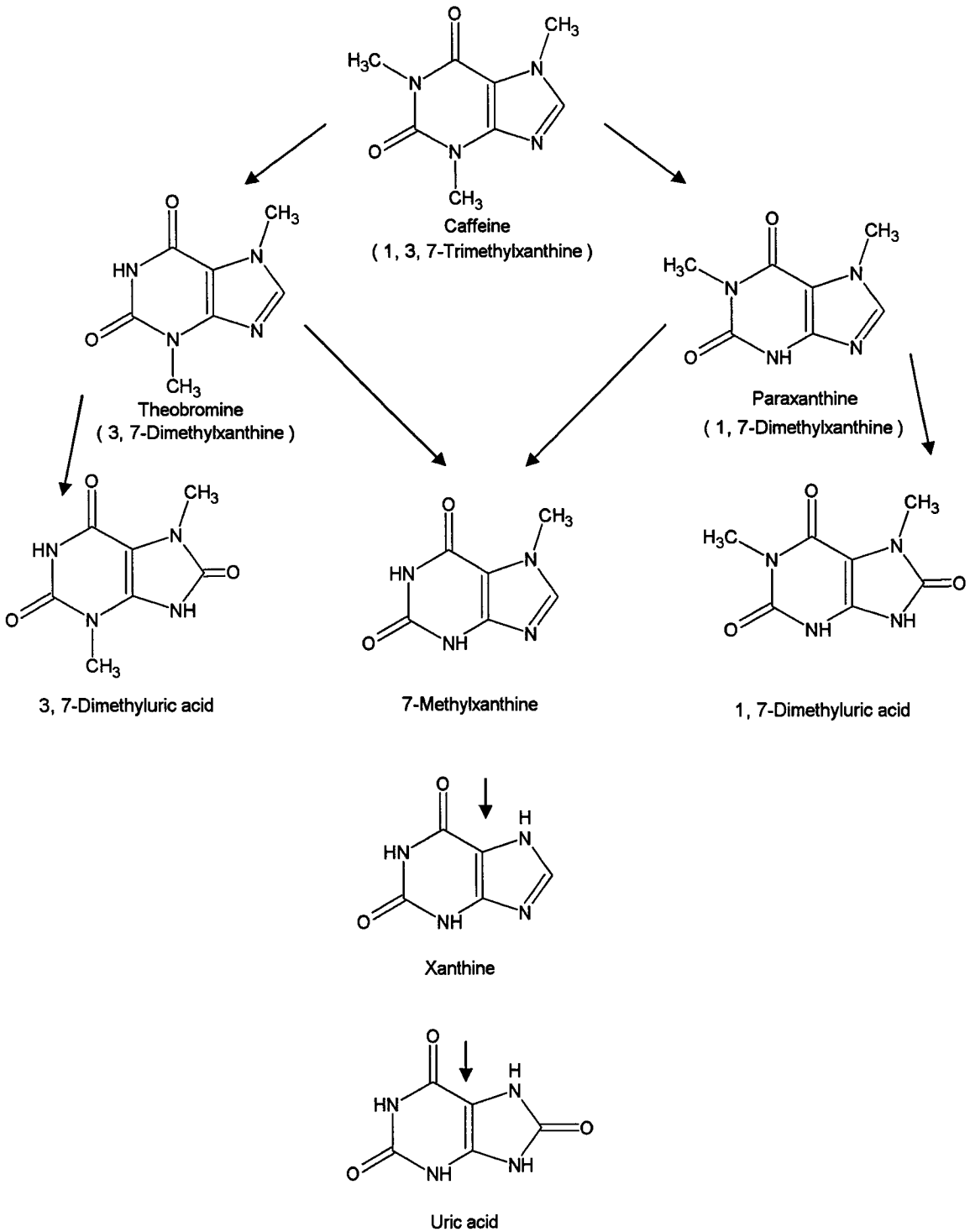


Figure 1-5: Demethylation and oxidation of *N*-xanthines by *Pseudomonas putida* (BLECHER and LINGENS 1977).

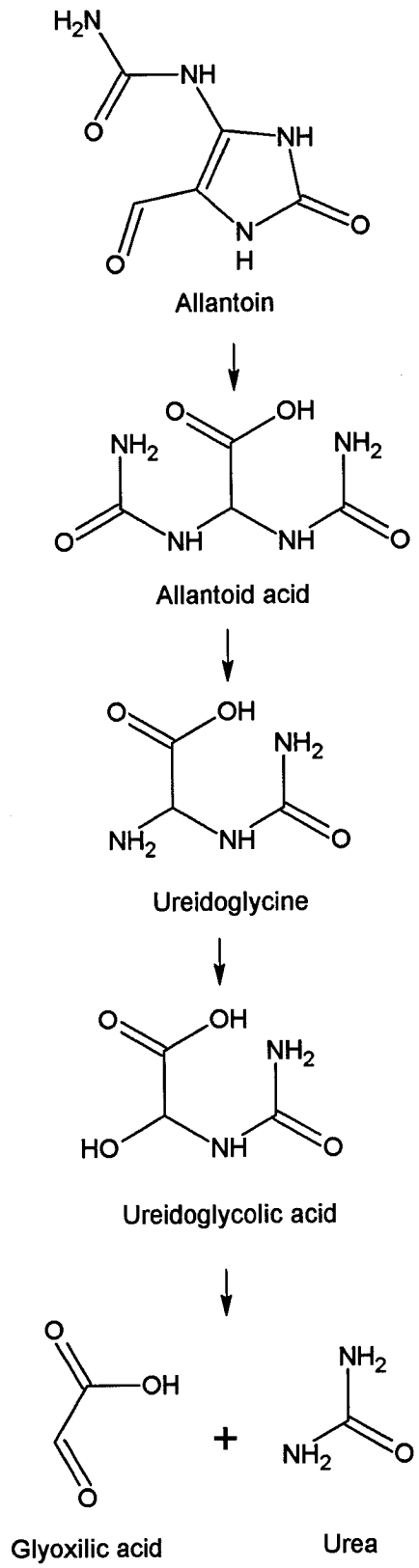


Figure 1-6: Purine breakdown by *P. putida.* (BLECHER and LINGENS 1977).

1.3.1 Temperature Effects on *P. putida*

For every organism there is a minimum temperature below which growth can no longer occur, an optimum temperature at which growth is most rapid, and a maximum temperature above which growth is not observed. The optimum temperature is most commonly found nearer to the maximum than to the minimum temperature (BROCK 1979).

As temperature rises, chemical and enzymatic reactions in the cell proceed at more rapid rates (BROCK 1979). When temperature rises above the optimum, cellular decomposition reactions are favored (ALAGAPPAN and COWAN 2004), resulting in irreversible damage to proteins, nucleic acids, and other cellular components (BROCK 1979) producing reduced metabolic functions (ALAGAPPAN and COWAN 2004). Bacterial membranes for example, undergo substantial alterations in their properties, both functional and structural, with increasing temperature. This fact, along with inactivation of some cytoplasmic enzymes, results in a lack of control over the materials which permeate into and out of the cells, producing an overall drop in cellular activity before complete thermal inactivation occurs (ALAGAPPAN and COWAN 2004).

Pseudomonas putida IF-3 was reported by Koide (KOIDE et al. 1996) to grow, using caffeine as it sole source of carbon and nitrogen, at 5°C, 25°C, and 37°C; its optimal growth temperature was found to be 30°C. The bacterium was not able to grow at 42°C, and death resulted after a 30 minute exposure to 60°C (KOIDE et al. 1996).

Blecher (BLECHER and LINGENS 1977) found that *Pseudomonas putida* C1 grown on caffeine as sole source of carbon and nitrogen had an optimal growth temperature between 25°C and 30°C; also no growth was observed at 40°C. However, despite the lack of growth at 40°C, lypases (LEE and RHEE 1993) and esterases (OSAKI and SAKIMAE 1997) from *Pseudomonas putida* have been reported to be stable up to 70°C.

1.3.2 Cell Free Extracts of *P. putida*

To further elucidate the caffeine degradation process by *Pseudomonas putida*, experiments with cell free extracts have been performed. Cell lysis has been achieved by grinding with alumina (BLECHER and LINGENS 1977), bead milling (ASANO et al. 1994) and sonication (HOHNLOSER et al. 1980) (SIDESO et al. 2001) (YAMAOKA-YANO and MAZZAFERA 1999). Separation of cell debris was performed in these studies by centrifugation at 4°C. The lysis efficiency was not reported in these studies. The supernatant was always used for caffeine degradation assays (BLECHER and LINGENS 1977), (ASANO et al. 1994), (HOHNLOSER et al. 1980), (SIDESO et al. 2001), (YAMAOKA-YANO and MAZZAFERA 1999), and cell debris was reported to have no detectable activity (ASANO et al. 1994), (HOHNLOSER et al. 1980).

It has been shown that the presence of NADH is absolutely necessary for caffeine degradation activity in cell free extracts of *P. putida* (HOHNLOSER et al. 1980; ASANO et al. 1994). The reaction is shown in Figure 1-7.

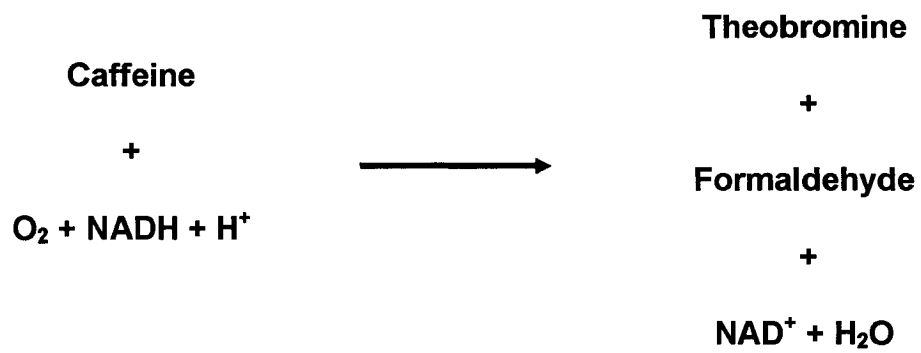


Figure 1-7: Caffeine demethylation by cell free extracts of *Pseudomonas putida* (HOHNLOSER et al. 1980; ASANO et al. 1994).

Asano and coworkers hypothesized that the demethylation of caffeine is catalyzed by several enzymes (ASANO et al. 1993). Asano was able to isolate three different enzyme fractions from cell free extracts of *P. putida;* each fraction had different substrate specificity for caffeine, caffeine and theobromine, and caffeine and 7-methylxanthine respectively (ASANO et al. 1994). Hohnloser (HOHNLOSER et al. 1980) and Asano (ASANO et al. 1994) also found that formaldehyde dehydrogenases were present in cell free extracts; these enzymes catalyze the reaction shown in Figure 1-8, where the formaldehyde produced in the breakdown of caffeine is oxidized to formic acid, and NAD^+ is reduced back to NADH.

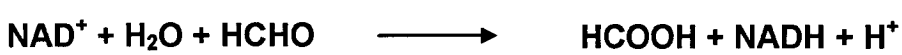


Figure 1-8: Oxidation of formaldehyde by cell free extracts of *Pseudomonas putida* (HOHNLOSER et al. 1980).

Hohnloser (HOHNLOSER et al. 1980) also showed that addition of certain divalent ions to reaction mixtures increased cell free extract caffeine degradation rates (Table 1-1).

System	Percent Activity
No divalent ion addition	100 %
Co^{2+}	153 %
Cu^{2+}, Fe^{2+}	115 %
Mg^{2+}	100 %
Zn^{2+}, Sn^{2+}	90 %

Table 1-1: Effect of divalent ions on caffeine degrading activity of cell free extracts from *Pseudomonas putida* (HOHNLOSER et al. 1980).

Cell free extract caffeine degrading activity was reported to be lost within few hours of preparation even when the cell free extract was stored at 4°C (ASANO et al. 1994) (SIDESO et al. 2001). Free water has been proposed to be detrimental to the stability of the demethylases (SIDESO et al. 2001). By using a cryoprotectant, and freeze-drying cell free extracts to less than 5 % water content, Sideso *et al.* were able to retain 50 % of the initial activity in cell free extracts, when stored either at room temperature or at 4°C, for up to three months (SIDESO et al. 2001).

SDS-PAGE analyses have been performed on cell free extracts of *P. putida* grown on caffeine as it sole source of carbon and nitrogen (ASANO et al. 1994), (OGUNSEITAN

1996), (SIDESO et al. 2001). A distinct band has been observed at 40 kDa by Ogunseitan (OGUNSEITAN 1996), Asano (ASANO et al. 1994), and Sideso (SIDESO et al. 2001). Asano also reported a secondary band at 36.6 kDa (ASANO et al. 1994).

Blecher (BLECHER and LINGENS 1977) reported the presence of cytochrome oxidases in *Pseudomonas putida* grown with caffeine as its sole source of carbon. Ogunseitan (OGUNSEITAN 2002) also found that growth on caffeine induced the over-expression of P450-type cytochrome and peroxidase enzymes in *Pseudomonas putida*. Animals, plants and microorganisms contain cytochromes P450 (PORTER and COON 1991). The substrates for cytochrome P450 encompass a host of xenobiotics, including substances that occur biologically but are foreign to living organisms, antibiotics, synthetic organic chemicals, and a variety of steroids (PORTER and COON 1991). P450 may also promote *N*-demethylation reactions (WHITE and COON 1980). The most completely characterized P450 is the soluble, bacterial P450, $P450_{cam}$ (PETERSON and GRAHAM-LORENCE 1995). $P450_{cam}$ is a cytosolic cytochrome from *Pseudomonas putida* (PORTER and COON 1991) which catalyzes the hydroxylation of camphor (PETERSON and GRAHAM-LORENCE 1995). The P450-type cytochrome and peroxidase enzymes found by Ogunseitan (OGUNSEITAN 2002) proved to be different from $P450_{cam}$ (OGUNSEITAN 2002).

1.4 Factors that Affect Enzymatic Activity

Protein structure is stabilized by weak forces; as a result, enzymes are energetically poised near several alternative and less biologically active configurations (BAILEY and OLLIS 1986). The free energy difference between the native and completely denatured state is very small, although several intermediate energy barriers must be overcome in order to go from one conformation to the other. Consequently, native protein structure is only marginally stable (BAILEY and OLLIS 1986). As a general rule of thumb: if the enzyme is surrounded *in vitro* with essentially the same environment it enjoys *in vivo*, it will be active. If any parameter of its environment is altered significantly, loss of activity is likely to occur (BAILEY and OLLIS 1986).

Factors such as water concentration, ionic strength, protein concentration, and the protective effect of substrate, and other substances, may have a considerable effect on the inactivation rate of enzymes (DIXON and WEBB 1979). Water concentration and ionic strength have a direct influence on thermodynamic water activity (SMITH et al. 1996). Water activity controls biological effects, which arise from the complex interplay of water, macromolecules, and other solutes (BATEMAN and WHITE 1963).

Mechanical forces can also disturb the elaborate shape of an enzyme molecule to such a point that deactivation occurs. Included among such forces are forces created by flowing fluids (BAILEY and OLLIS 1986).

On the whole, it is not the individual factors, but their combinations that determine rates of enzyme deactivation (BAILEY and OLLIS 1986). What is more, it is the combined effect of exposure time and intensity of these denaturing factors that will determine the extent of deactivation of enzymes (BAILEY and OLLIS 1986).

1.4.1 Temperature

Two different effects of augmenting temperature operate simultaneously: the increase in the initial velocity or true catalytic activity of the enzyme, and the destruction of the enzyme at higher temperatures (DIXON and WEBB 1979). Very few enzymes can survive prolonged heating, yet thermal deactivation of enzymes may be reversible (BAILEY and OLLIS 1986).

Generally, enzymatic denaturation is not a catastrophic event, but rather occurs gradually (REINER 1969). For many proteins, denaturation begins to occur at 45°C to 50°C, and is severe at 55°C. As temperature increases, the atoms in the enzyme molecule eventually acquire sufficient energy to overcome the weak interaction holding the protein structure together, and deactivation follows (BAILEY and OLLIS 1986). However, the impact of temperature on the apparent kinetics of enzimatically mediated reactions may be due to many other causes besides an effect on the stability of the enzyme (DIXON and WEBB 1979). Some of these causes are: i) an effect on the velocity of breakdown of the enzyme-substrate complex, ii) an effect on the affinity of the substrate for the active site; iii) an effect on the pH functions of any or all of the components, due to an alteration of their pK_as, which is determined by heats of ionization; iv) an effect on the affinity of the

enzyme for activators or inhibitors, if any; v) a transfer of rate-limiting function from one enzyme to another, in a system involving two or more enzymes with different temperature coefficients; or vi) even a change in concentration of dissolved O_2 (DIXON and WEBB 1979).

2. Methods and Materials

2.1 Cell Culture

The strain of *P. putida* used in this study was *Pseudomonas putida* IF-3 (KOIDE et al. 1996). This bacterium was originally isolated from soil (KOIDE et al. 1996) and the strain was selected for its tolerance to high caffeine concentrations, and for its ability to use caffeine as it sole source of carbon and nitrogen (KOIDE et al. 1996). The bacterium was stored at -70°C (Model ULT1386, Revco) in 1.5 mL mini-centrifuge vials. The vials contained a 1:1 (v / v) mixture of growth medium (Table 2-1) and 40% glycerol. To revive an inoculum, the contents of one vial were thawed and transferred to a 500 mL, sterile shake flask containing 150 mL of medium (Table 2-1). The flask was then covered with a sponge cap and incubated for 72 hours, at which time a 1 mL sample was used as an inoculum for a new flask containing fresh sterile medium.

Incubation was done at room temperature, at 200 rpm, in a rotary incubator shaker with a 5 cm stroke (New Brunswick Scientific, Model G-25). Prior to inoculation, shake flasks and caffeine-free media were steam sterilized separately for 30 min at 121°C and 17 psig (AMSCO, Model SG-116 or AMSCO, Model 3021-S). Immediately before inoculating, 150 mL of caffeine-free medium was poured into a sterile shake flask, and 1.5 g of caffeine were added aseptically. After complete dissolution of caffeine, flasks were inoculated with 1 mL samples from 3-day old shake flask cultures. The culture was maintained by repeating this operation every three days.

Component	g / L
Caffeine	10
$CaCl_2$	0.08
KCl	0.37
$MgSO_4 \cdot 7H_2O$	0.205
Na_2HPO_4	0.67
$FeCl_3$	0.71
	µg / L
H_3BO_3	10
$Co(NO_3)_2 \cdot 6H_2O$	15.9
$CuSO_4$	10
$MnSO_4 \cdot H_2O$	11.2
$NaMoO_4 \cdot 2H_2O$	11.8
$ZnSO_4 \cdot 7H_2O$	15

Table 2-1: Defined growth medium used for shake flask cultures of *P. putida* IF-3.

2.2 Analytical Procedures

2.2.1 Biomass Concentration

2.2.1.1 Dry Weight

Biomass from the shake flasks was measured by dry weight. A 15 mL sample of a shake flask was centrifuged at 12,500 x g, for 15 minutes, at 4°C (International Equipment Co., model B-22M). The supernatant was discarded, 15 mL of distilled water were added, and the pellet was re-suspended prior to centrifugation. This was done twice. After the second centrifugation step the pellet was re-suspended in 2 mL of distilled water and poured into a desiccated, aluminum dish of known weight. The dish was then dried at 105°C overnight to a constant weight. The dry weight determination was always done in duplicate and reported in grams of dry cells per liter (g cells / L).

2.2.1.2 Optical Density

Optical density was used to distinguish the growth phase at the time of harvest. Measurements were used to ensure cultures were well in the stationary phase of growth by comparison with a growth curve (Figure 2-1) for *Pseudomonas Putida* IF-3, growing in shake flasks, using the medium described in section 2.1. Optical density was measured at 600 nm using a double-beam, UV spectrophotometer (Varian Inc, Model Bio 100 UV). Samples of 3 mL were taken from the shake flasks and diluted with sterile growth medium, if necessary, to obtain absorbance readings below 0.9. Sterile growth medium (Table 2-1) was used as the reference solution.

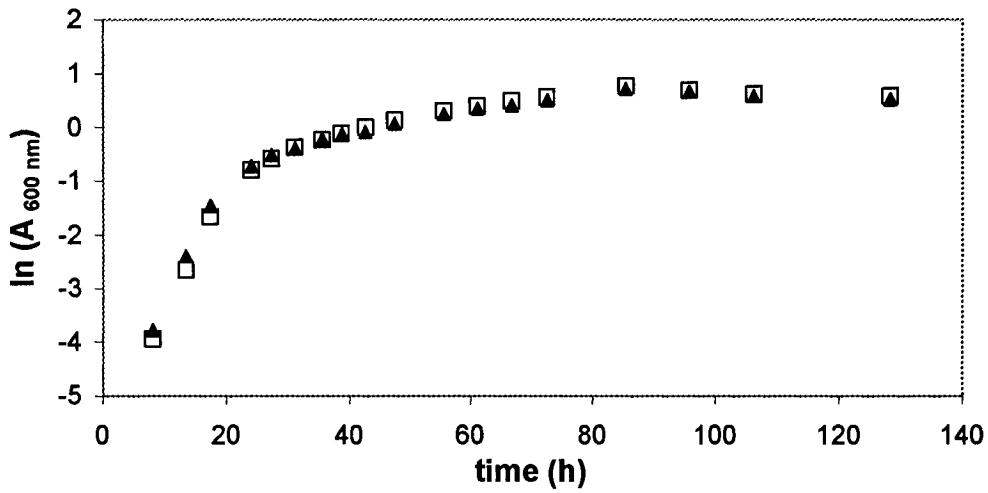


Figure 2-1: Growth curve for *Pseudomonas putida* IF-3 growing on caffeine at 25°C. Flask 1 (□) and flask 2 (▲). Samples of 3 mL were taken from the shake flasks and diluted with sterile growth medium, if necessary, to obtain absorbance readings below 0.9. Absorbance was measured at 600 nm, at 22°C.

2.2.2 NADH

NADH was measured by absorbance at 340 nm using a double-beam, UV spectrophotometer (Varian Inc, Model Bio 100 UV). A 1.49 mM solution of β-NADH disodium salt (98 %, Sigma-Aldrich) was prepared in TRIS (99.8 %, Bio-Rad Laboratories) buffer adjusted to pH 7.5 with 3 N HCl, and serial dilutions were prepared to obtain a calibration curve (Figure 2-2).

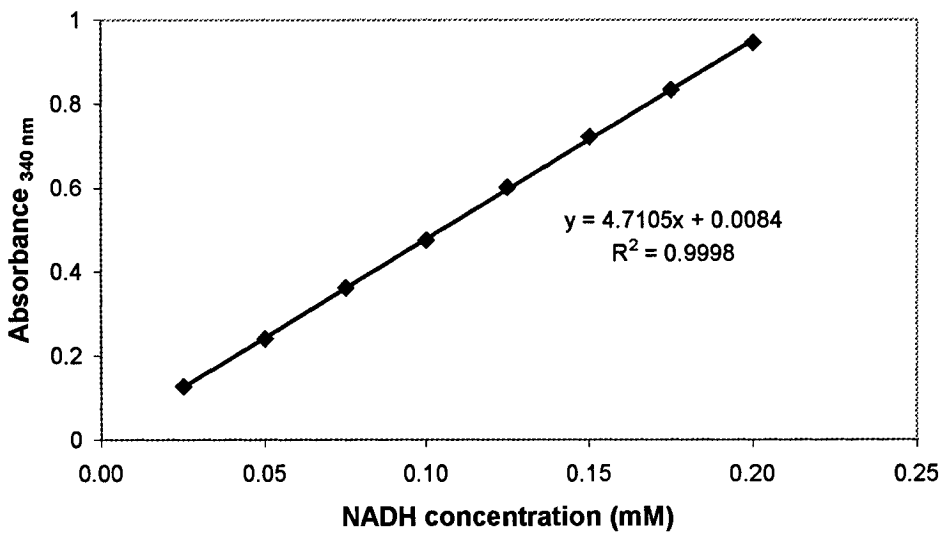


Figure 2-2: Absorbance calibration curve for NADH. NADH was detected at 340 nm using a double-beam spectrophotometer.

Samples were randomized before the readings. TRIS buffer was used as the reference solution. Seven replicates of a solution with a known concentration of NADH were analyzed to assess the error associated with the calibration curve. With 95 % confidence, the error was found to be ± 0.0004 mM, and the variation coefficient was 0.011.

2.2.3 Caffeine

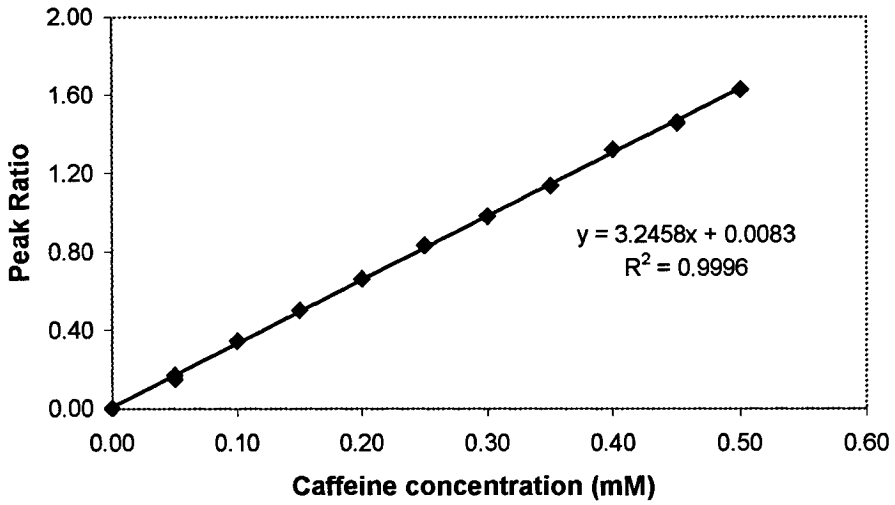


Figure 2-3: HPLC caffeine calibration curve. The peak ratio is obtained by dividing the area of the caffeine peak by the area of the internal standard peak in an HPLC chromatogram (Figure 2-4).

Caffeine concentration was measured by high performance liquid chromatography (HPLC) (Agilent Technologies Inc., 1100 series). The mobile phase used was 80 parts of 1 % aqueous acetic acid to 20 parts of methanol (v / v) flowing at 1 mL / min (ASANO et al. 1994). The internal standard was 8-chlorotheophylline (98 %, Sigma-Aldrich) as suggested by Jodynis-Liebert (JODYNIS-LIEBERT and MATUSZEWSKA 1999). The final concentration of the internal standard in each sample was always 0.25 mM. Separation was achieved using a 4.6 x 250 mm, C-18 column with 5 µm packing (Zorbax, model Eclipse XDB-C18), and the effluent was monitored with a variable wavelength UV detector set at 273 nm. Samples were always filtered through disposable 0.2 µm syringe filters (Whatman, 13 mm nylon membrane), and randomized before the

analysis. Solutions with known concentrations of caffeine were always analyzed every 10^{th} sample for control purposes.

To obtain a calibration curve for caffeine, a 4.1 mM solution of caffeine was prepared in TRIS (99.8 %, Bio-Rad Laboratories) buffer, adjusted to pH 7.5 with 3 N HCl, and serial dilutions were made and analyzed (Figure 2-3). Seven replicates of a solution with a known concentration of caffeine were analyzed to assess the error associated with the calibration curve. With 95 % confidence, the error was found to be ±0.003 mM, and the variation coefficient was 0.0004. With this setup theobromine could also be detected. A typical chromatogram is shown in Figure 2-4.

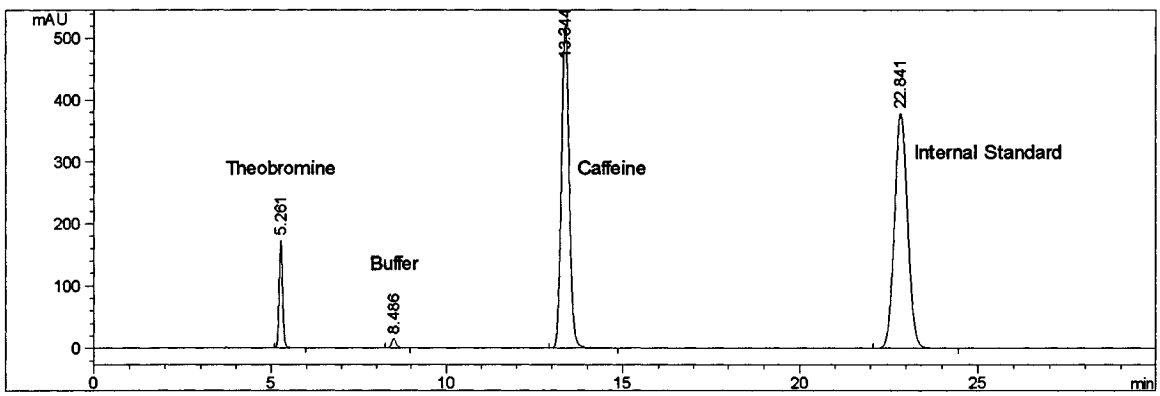


Figure 2-4: Sample HPLC chromatogram for caffeine analysis.

2.2.4 Protein Assay

Protein content in cell free extracts was determined using a modification of the Lowry (LOWRY et al. 1951) assay (Bio-Rad 2002). This assay is based on the reaction between protein, and an alkaline copper tartrate solution, and then with Folin reagent (FOLIN and CIOCALTEAU 1927). Tyrosine and tryptophan in protein are mainly responsible for the development of a characteristic blue with maximum absorbance at 750 nm (PETERSON 1979).

Samples of 100 µL were mixed in a test tube with 500 µL of an alkaline copper tartrate solution (Reagent A, Bio-Rad) and then with 4 mL of dilute Folin reagent (Reagent B, Bio-Rad). The mixture was vortexed for 30 seconds, immediately after adding the Folin reagent. After 30 minute incubation at 22°C, absorbance was measured at 750 nm using a double-beam, UV spectrophotometer (Varian Inc, Model Bio 100 UV).

To obtain a calibration curve, a fresh solution (1 g / L) of bovine serum albumin (BSA) was prepared in TRIS (99.8 %, Bio-Rad Laboratories) buffer, adjusted to pH 7.5 with 3 N HCl, and serial dilutions prepared and analyzed. Samples were always analyzed in duplicate and TRIS (99.8 %, Bio-Rad Laboratories) buffer, adjusted to pH 7.5 with 3 N HCl, was used as the reference solution.

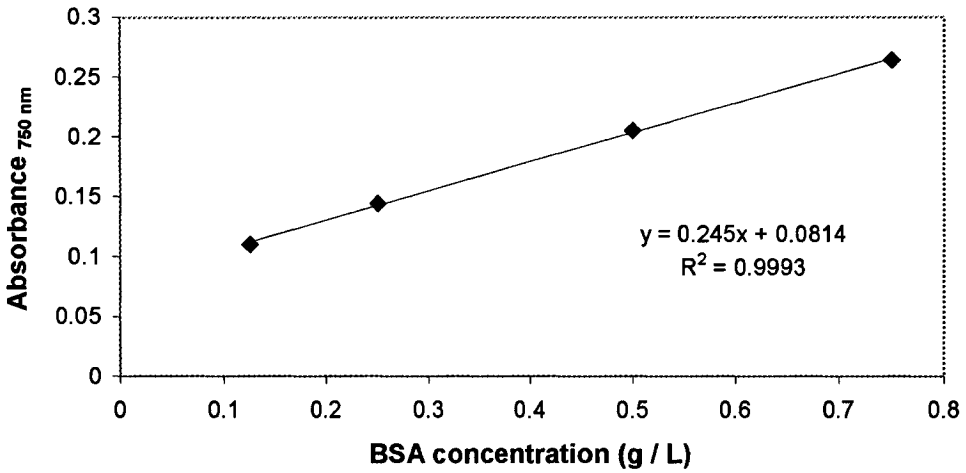


Figure 2-5: Protein content calibration curve. Bovine serum albumin was used as a standard and absorbance was measured at 750 nm, at 22°C.

2.2.5 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The proteins present in cell free extracts were separated by size using SDS-PAGE. Gels of 0.75 mm were run on a discontinuous buffer system. TRIS-glycine (pH 8.3) was used as the electrode running buffer, TRIS · Cl (pH 6.8) as the stacking gel buffer, and TRIS · Cl (pH 8.8) as the resolving gel buffer (LAEMMLI 1970). Gels were cast following the gel cassette manufacturer's instructions (Bio-Rad 2003). The gel formulation used is shown in Table 2-2 (SAMBROOK et al. 1989).

Samples of 10 µL were mixed with 15 µL of sample buffer (Table 2-3). The mixture was then placed in a water bath and kept at 95°C for 4 minutes (SAMBROOK et al. 1989). A volume of 15 µL was loaded per well. A molecular weight ladder of 14.4 to 97.4 kDa (Table 2-4) (Bio-Rad Laboratories) was used as a standard.

	Volume (mL)
H_2O	3.4
30 % Acrylamide 0.8 % N'N'-bis-methylene-acrylamide	4.0
Gel Buffer	2.5
10 % (w/v) SDS	0.1
10 % Ammonium Persulfate	0.1
TEMED	0.004

Table 2-2: 12 % acrylamide gel formulation (SAMBROOK et al. 1989). Half the amount of TEMED was used for stacking gels.

	Volume (mL)
H_2O	3.55
Stacking gel buffer	1.25
Glycerol	2.5
10 % (w / v) SDS	2.0
0.5 % (w / v) bromophenol blue	0.2

Table 2-3: Sample buffer (SAMBROOK et al. 1989). A volume of 50 µL of β-Mercaptoethanol was added to 950 µL of sample buffer prior to use.

Protein	Molecular Weight (kDa)
Phosphorylase b	97.4
Serum albumin	66.2
Ovalbumin	45.0
Carbonic anhydrase	31.0
Trypsin inhibitor	21.5
Lysosyme	14.4

Table 2-4: Molecular weight standard (Bio-Rad Laboratories).

Electrophoresis was carried inside a Mini-PROTEAN 3 electrode module (Bio-Rad Laboratories) using 200 V, constant voltage. When electrophoresis was completed, gels were stained by immersion in a Coomasie Brilliant Blue solution (Table 2-5) for 4 hours, and destained by soaking the gels in a 30 % (v / v) methanol, 10 % (v / v) acetic acid, aqueous solution for two hours (SAMBROOK et al. 1989).

Coomasie Brilliant Blue R250	0.25 g
Methanol	45 mL
H_2O	45 mL
Glacial Acetic Acid	10 mL

Table 2-5: Staining solution used to treat gels after electrophoresis (SAMBROOK et al. 1989). The solution was filtered through a Whatman No.1 filter prior to use.

Gels were then visualized under white light, using a transilluminator equipped with a digital camera (Bio-Rad Laboratories). Band analysis was performed with image analysis software (Quantity One, Version 4.2.2, Bio-Rad Laboratories).

2.3 Resting Cell Suspension Preparation

Cells were always harvested after approximately 3 days of growth in shake flasks with caffeine as their sole source of carbon and nitrogen as explained in Section 2.1. Optical density was measured immediately before harvesting cells, as described in section 2.2.1.2, and the culture age and absorbance were recorded. The result was compared with the growth curve in Figure 2-1 in order to ensure the culture was in stationary phase.

A volume of 100 mL of liquid culture (0.48 to 0.96 g / L, dry weight) from the 3-day old shake flask culture was then equally distributed into 4 centrifuge tubes, and centrifuged at 12,500 x g for 15 minutes at 4°C. The supernatant was discarded, and the pellets were washed twice with caffeine-free medium, and resuspended in 4 mL of TRIS (99.8 %, Bio-Rad Laboratories) buffer adjusted to pH 7.5 with 3 N HCl. The four portions were then recombined, and the resulting 16 mL were used immediately for either resting cell experiments or for cell lysis.

2.4 Cell Free Extract Preparation

The 16 mL aliquot of fresh resting cell suspension, obtained as described in section 2.3, was lysed by fluid shearing (TOGASAKI et al. 2000) (Bioneb Cell Disruption system, Glas-Col Inc.) for 20 minutes. The fluid stress was driven by a nitrogen (100 psi) venturi to achieve 20 mL / min flow rate, in closed-cycle mode.

Lysis products were separated by either filtration or centrifugation. For filtered cell free extracts, freshly lysed cell suspensions were filtered through sterile, 0.2 μm syringe filters (Corning Inc., 26 mm, SFCA membrane) and the filtrate used immediately for the caffeine degradation assays. When centrifuged cell free extracts were being prepared, the lysed cell suspensions were centrifuged at 18,000 x g for 60 minutes at 4°C. The supernatant was used immediately for the caffeine degradation assays (Section 3.1.1), and the pellets discarded.

2.5 Caffeine Degradation Rates

2.5.1 Cell Free Extract Caffeine Degradation Rates

A method to quantify cell free extract caffeine degradation rates was developed as described in section 3.1.1. Cell free extract caffeine degradation experiments were carried at 22, 33, 42 and 50°C.

2.5.2 Resting Cell Caffeine Degradation Rates

A 25 mL volume of a TRIS buffer (pH 7.5), containing 0.15 mM NADH, and 0.45 mM caffeine was placed in a water bath, kept at the temperature of the experiment. Air was then bubbled through the solution for 20 minutes in order to assure O_2 saturation. A 2 mL volume of this O_2 saturated solution was transferred to a 4.5 mL, propylene disposable cuvet, and the latter placed inside the water bath. After one minute had elapsed, 1 mL of resting cell suspension, prepared as described in section 2.3, was added. Initial concentrations are shown in Table 2-6.

	Concentration
Resting Cells	1 to 2 g cells / L
NADH	0.1 mM
Caffeine	0.3 mM

Table 2-6: Initial concentrations for resting cell, caffeine degradation experiments

The caffeine degradation reaction was stopped after two minutes by filtering through a sterile, 0.2 μm filter (Corning Inc., 26 mm, SFCA membrane), and the filtrate was kept at 4°C until analyzed for caffeine. Caffeine content in the filtrate did not change over time. Caffeine degradation rates were calculated by dividing the change in caffeine concentration by the time it took to obtain the filtrate. Rates were then normalized by dry cell concentration. Resting cell caffeine degradation experiments were carried at 22, 33, 42 and 50°C following the method described above.

2.6 Stability Measurement

The activity of resting cells and cell free extracts was monitored for one hour. This was done at 22°C and also at 50°C. Three assays were performed for cell free extracts and for resting cells throughout the one hour period, for each temperature. The assays were done at the same temperature. The first assay was done immediately after preparation of either cell free extracts or resting cell suspensions, as described in section 2.5.

The remaining cell free extract or resting cell suspension was kept inside a water bath at the temperature of the experiment, and a second assay performed 15 minutes after the first one. After this second assay, the remaining cell free extract or resting cell suspension was kept inside a water bath at the temperature of the experiment, and a third assay was done 60 minutes after the first one. The second and third assays were performed according to the methods described in section 2.5.

2.7 Statistics

The error reported in section 2.2.2 and section 2.2.3 was calculated by multiplying the standard deviation by the appropriate Student-*t* cumulative probability. The variation coefficient reported in section 2.2.2, section 2.2.3, and section 3.2 was calculated by normalizing the standard deviation by the mean.

3. Results

3.1 Development of Analytical Methods

3.1.1 Cell Free Extract Caffeine Degradation Rates

The mechanism of demethylation of caffeine by cell free extracts of *P. putida* is shown in (Figure 1-7). Knowing that NADH is consumed during the reaction, together with the fact that NAD^+ does not absorb at 340 nm, UV-spectrophotometry seemed to be an adequate technique to follow the demethylation reaction. The disappearance of NADH could then be correlated to caffeine degradation.

To test this hypothesis 3 mL of TRIS buffer (pH 7.5) saturated with oxygen (0.3 mmol of O_2 / L at room temperature), containing 0.10 mM NADH, and fresh cell free extract was transferred to a 4.5 mL, propylene disposable cuvet. Absorbance was monitored at 340 nm, with TRIS buffer (pH 7.5) as the reference solution. Two variations of this experiment were also done. Table 3-1 summarizes the conditions for each experiment.

Experiment	Caffeine	NADH	Filtered Cell Free Extract
1	0.3 mM	0.1 mM	Added
2	0.3 mM	0.1 mM	Not Added
3	0	0.1 mM	Added

Table 3-1: Conditions for cell free extract, NADH consumption experiments. TRIS buffer (pH 7.5) was added instead of cell free extract in experiment 2.

When no cell free extract was present, no NADH oxidation was observed (Figure 3-1). However, NADH was oxidized by cell free extracts even in the absence of caffeine (Figure 3-1). As a result, NADH consumption could not be directly correlated to caffeine consumption.

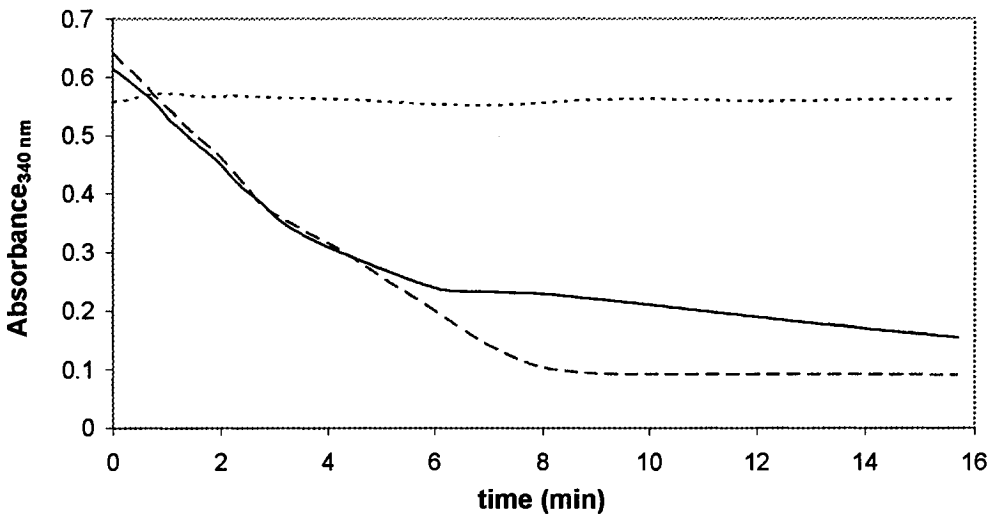


Figure 3-1: NADH consumption of cell free extracts in the presence and absence of caffeine. (····) Control, adding buffer to a buffer-NADH solution; (—) cell free extract added to a buffer-NADH solution; (----) cell free extract added to a buffer-NADH-caffeine solution.

To elucidate the dependence of caffeine removal on NADH, 3 mL of TRIS buffer (pH 7.5), saturated with oxygen (0.3 mmol of O_2 / L at room temperature), containing 0.10 mM NADH, and fresh cell free extract was transferred to a 4.5 mL, propylene disposable cuvet. Absorbance was monitored at 340 nm until a plateau was reached (Figure 3-2). At 8.3 minutes the solution was spiked with a caffeine solution. It was found that no caffeine was degraded. This experiment was done for filtered cell free extracts as well as for centrifuged cell free extracts.

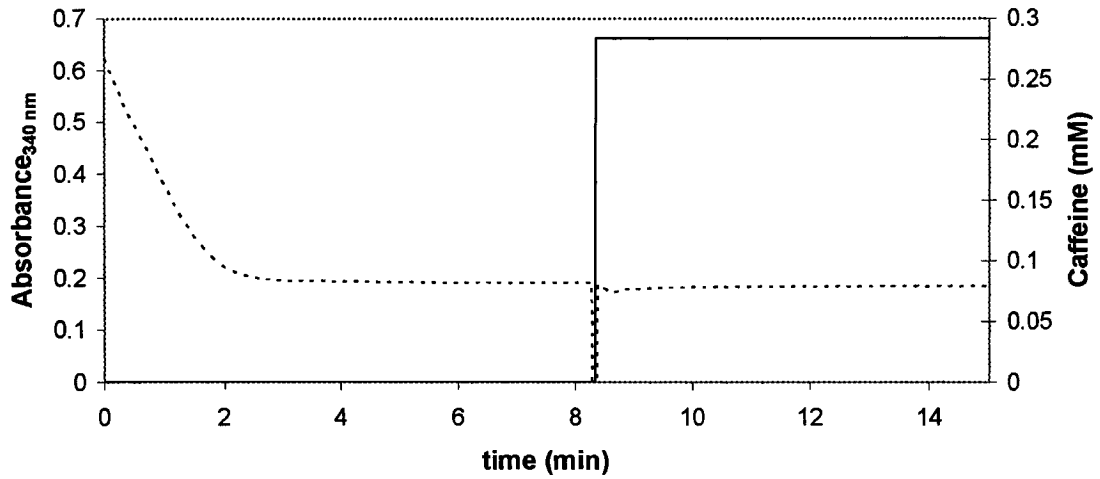


Figure 3-2: Caffeine spike after NADH was consumed by cell free extracts in the absence of caffeine. No change observed in the caffeine concentration. (....) Absorbance measured at 340 nm; (—) caffeine concentration.

With the certainty that no caffeine could be degraded by cell free extracts once a minimum level in absorbance was reached (Figure 3-2) and maintained (Figure 3-2) the caffeine degradation rates were determined in the following manner.

A 25 mL volume of TRIS buffer (pH 7.5), containing 0.15 mM NADH, and 0.45 mM caffeine was placed in a water bath, kept at the temperature of the experiment. Air was then bubbled through the solution for 20 minutes in order to assure O_2 saturation. A 2 mL volume of this O_2 saturated solution was put in a 4.5 mL, polystyrene disposable cuvet. Absorbance was monitored at 340 nm, at the temperature of the experiment, and TRIS buffer (pH 7.5) was used as the reference solution. After one minute had elapsed, 1 mL of freshly prepared cell free extract (section 2.4) was added. Initial concentrations are shown in Table 3-2.

	Concentration
CFE	0.2 to 0.7 g protein / L
NADH	0.1 mM
Caffeine	0.3 mM

Table 3-2: Initial concentrations for cell free extract, caffeine degradation experiments

Once a minimum level of NADH was reached and maintained for at least 5 minutes (Figure 3-3), the cuvet was taken out of the spectrophotometer and kept at 4°C until analyzed for caffeine.

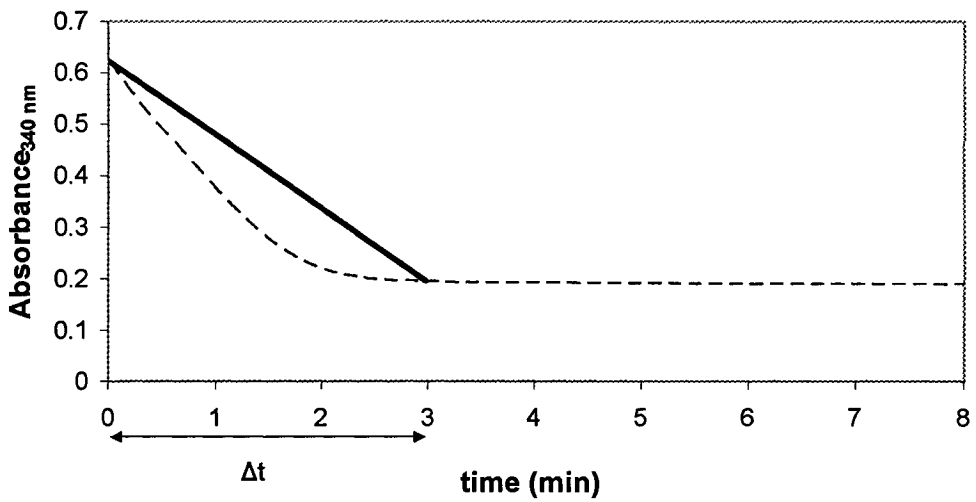


Figure 3-3: Example of how to obtain cell free extract caffeine degradation times using an NADH consumption curve. (- - -) absorbance at 340 nm; (—) drawn to determine Δt.

A line was then drawn as in Figure 3-3 to determine the reaction rate. Estimated in this manner, the reaction time (Δt in Figure 3-3) corresponds to a conservative estimate of the time it took the system to degrade caffeine. It could be an overestimate however, for complete caffeine degradation could have been achieved any point prior. Thus, the reported caffeine degradation rates for cell free extracts are conservative estimates.

The rates were then calculated by dividing the change in caffeine concentration by the reaction time, and normalized by the protein content (Section 2.2.4), or by the equivalent dry cell concentration (Section 3.1.3). Cell free extract caffeine degradation experiments were carried at 22, 33, 42 and 50°C following the method described above.

3.1.2 Lysis Efficiency

The extent of light transmitted through a sample of culture media is a function of cell density and the thickness of the chamber. By measuring turbidity it is possible to estimate cell density (SHULER and KARGI 1992). Using this observation, it was rationalized that the extent of lysis could be followed by measuring absorbance. For that purpose 0.1 mL samples from the cell suspension were taken at regular intervals during lysis, for a period of 25 minutes. Samples were diluted with 0.9 mL of buffer in a 1.5 mL quartz cuvet, and absorbance was measured at 600 nm. This experiment was repeated several times (Figure 3-4).

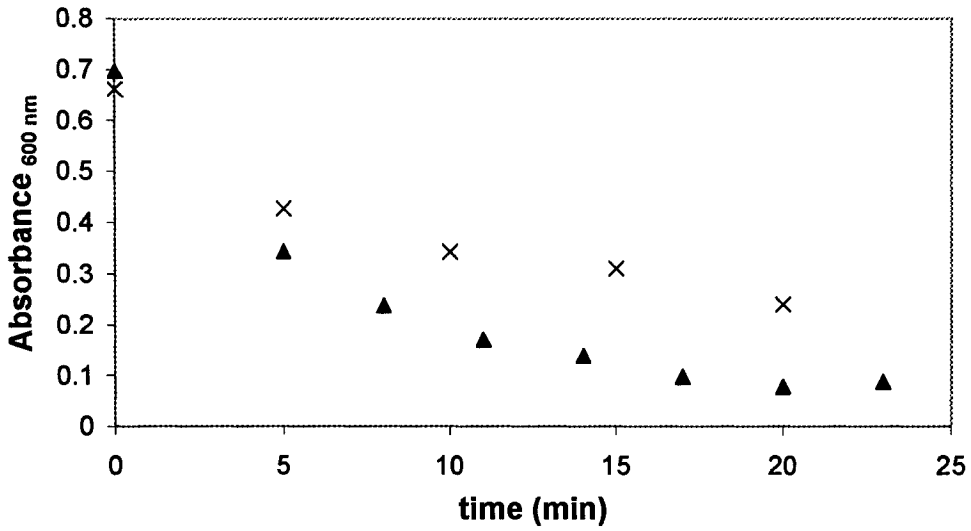


Figure 3-4: Lysis extent monitoring using absorbance. (×) Lysis preparation with $\mathbf{k} = 0.09\ min^{-1}$; (▲) lysis preparation with $\mathbf{k} = 0.14\ min^{-1}$.

The first step in quantifying lysis was to determine an adequate lysis time. Because the curves tended to flatten out after 15 minutes (Figure 3-4), every lysis procedure was carried for 20 minutes in order to make sure maximum cell disruption had occurred. Since sample volumes and geometries were always kept constant, measuring time was equivalent to the number of sample volumes passed through the nebulizer (turnover). Even though the absorbance curves tended to flatten out after 15 minutes, the curves would converge to different absorbances (Figure 3-4), indicating that lysis was more efficient in some preparations than in others.

Higher leveling points coincided with slower initial rates of lysis (Figure 3-4). Therefore, it was hypothesized that the initial rate of lysis could be modeled as a first-order process. Performing a mass balance around the nebulizer, and integrating the result yields:

Equation 3-1: $\ln(A_0 / A) = k t$

Where $\mathbf{A_0}$ is the initial absorbance, **A** is the absorbance at time "t", **k** is the first-order constant of the process (min^{-1}), and **t** is the time in minutes. With these definitions, **k** provides a measure of lysis efficiency, and a semi-log plot should yield a straight line with a slope equal to the rate constant (Figure 3-5). A process with a higher **k**-value would have a higher lysis-efficiency than a process with a lower **k**-value. After calculating rate constants for more than 90 lysis preparations it was found that the values approached 0.15 min^{-1}, although **k** never reached 0.15 min^{-1}. A **k**-value of 0.15 min^{-1} was assigned a lysis efficiency of 100 %. By finding **k** for each preparation, a lysis efficiency (η) could be assigned to each procedure.

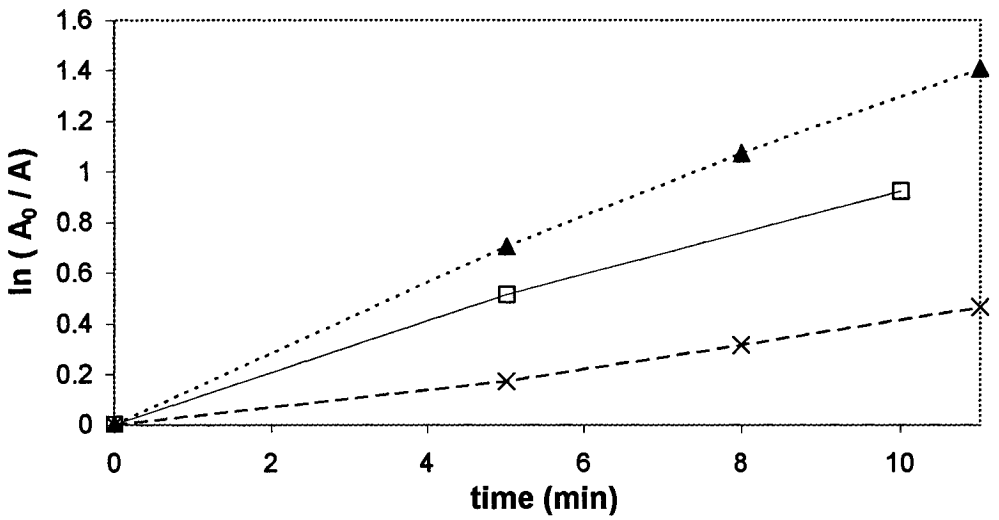


Figure 3-5: Example of plots used to show first-order behavior of absorbance during cell lysis; the slope of each line corresponds to the rate constant **k** (min^{-1}). (···▲···) Lysis preparation with **k** = 0.14; (—□—) lysis preparation with **k** = 0.10; (---×---) lysis preparation with **k** = 0.03.

3.1.3 Equivalent Dry Cell Concentration

It was necessary to find an equivalent to dry cell concentration for cell free extracts in order to compare the latter to resting cells, on the same basis. This equivalence stems from the lysis efficiency calculation (Section 3.1.2). Should $\eta = 1$ in a lysis step, then 100 % of the cells in suspension would have been lysed. If $\eta < 1$, then η corresponds to the fraction of cells that were lysed, and 1- η to the fraction of cells that were not lysed. The equivalent amount of dry cells in cell free extracts can then be found by multiplying η by the dry cell concentration in the suspension before lysis. In this manner, the fraction of cells that were not lysed (1- η) is not taken into account when calculating equivalent dry cell concentration in cell free extracts, since intact cells were removed from the lysate (section 2.4) .

3.2 Activity

Caffeine degradation rates were measured at four different temperatures (Figure 3-6) immediately after preparing either resting cell suspensions or filtered cell free extracts (section 2.5). Caffeine consumption rates are reported as mmol of caffeine degraded per gram of cells, per minute (mmol caffeine / g cells · min). In the case of cell free extracts, an equivalent amount of cells was used as described in section 3.1.3. Three replicates were done for resting cells and for cell free extracts at 22°C (Table 3-3).

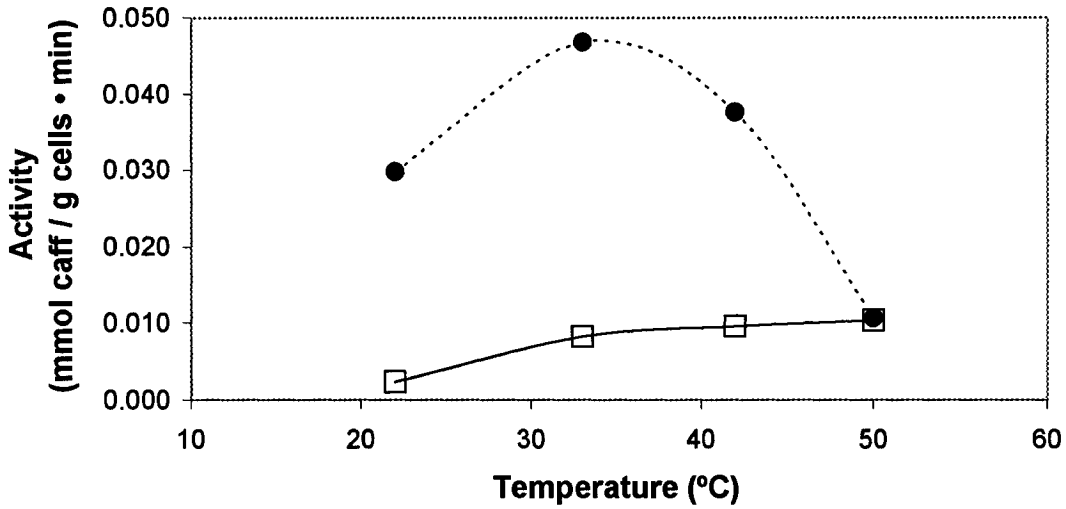


Figure 3-6: Filtered cell free extract (—□—) and resting cell (--•--) caffeine degradation rates at different temperatures. Three replicates were done for resting cells and for cell free extracts at 22°C, the variation coefficient was found to be 0.2 and 0.25 respectively.

	Mean Rate ($\times 10^2$ **mmol caffeine / g cells · min)**	**Standard Deviation** ($\times 10^2$ **mmol caffeine / g cells · min)**	**Variation coefficient** $\times 10^2$
Resting cells	2.98	± 0.6	0.20
Filtered Cell Free Extracts	0.24	± 0.06	0.25

Table 3-3: Caffeine degradation rates and statistics at 22°C. n = 3.

Cell free extracts showed a 400 % increase in activity when temperature was raised from 22°C to 33°C (Figure 3-6). Above 33°C activity kept increasing, although much more gradually, with a 20 % increase in activity observed between 33°C and 50°C (Figure 3-6).

A linear regression was done to check whether the rise in cell free extract activity with increasing temperature was statistically significant. The rise in activity with respect to temperature (mmol · g cells^{-1} · min^{-1} / °C) was significantly greater than zero ($P < 0.05$).

Resting cell caffeine degradation rates were found to be 12 times higher than cell free extract caffeine degradation rates at 22°C (Figure 3-6 and Table 3-3). Resting cell caffeine degradation rates passed through a maximum at 33°C, increasing by 57 % from 22°C to 33°C (Figure 3-6). In contrast to cell free extracts, the activity of resting cells started to decrease when temperature was raised above 33°C (Figure 3-6). Resting cell activity dropped by 77 % from 33°C to 50°C, and at 50°C resting cells and cell free extracts exhibited similar activities (Figure 3-6).

In order to associate the loss of activity with a particular step in the preparation of cell free extracts, caffeine degradation rates were measured at 22°C, immediately after cell lysis (Lysate in Table 3-4), and also after cell debris separation, either by centrifugation or filtration. The results are shown in Table 3-4. Results normalized with respect to resting cell activity are presented in Figure 3-7. After lysis, 38 % activity was retained, 21 % after separation by centrifugation, and 8 % after separation by filtration (Figure 3-7).

	Rate ($\times 10^2$ mmol caffeine / g cells · min)
Resting cells	2.98
Lysate	1.13
Centrifuged Cell Free Extracts	0.63
Filtered Cell Free Extracts	0.24

Table 3-4: Caffeine degradation rates for resting cells, lysate and centrifuged and filtered, cell free extracts, at 22°C.

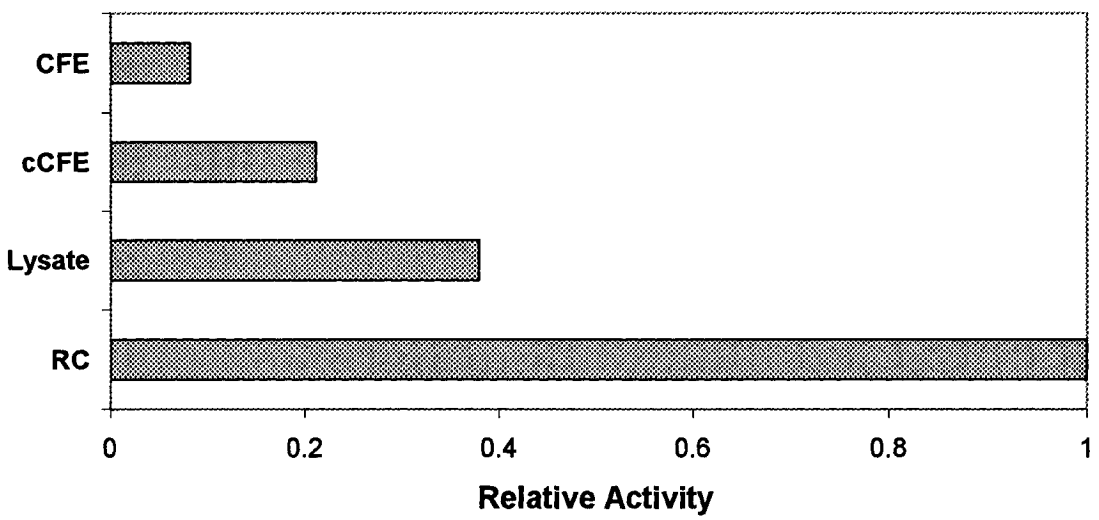


Figure 3-7: Relative activity retained by filtered cell free extracts (CFE), centrifuged cell free extracts (cCFE), lysate, and resting cells (RC). Activity was normalized with respect to resting cell activity.

3.3 Stability

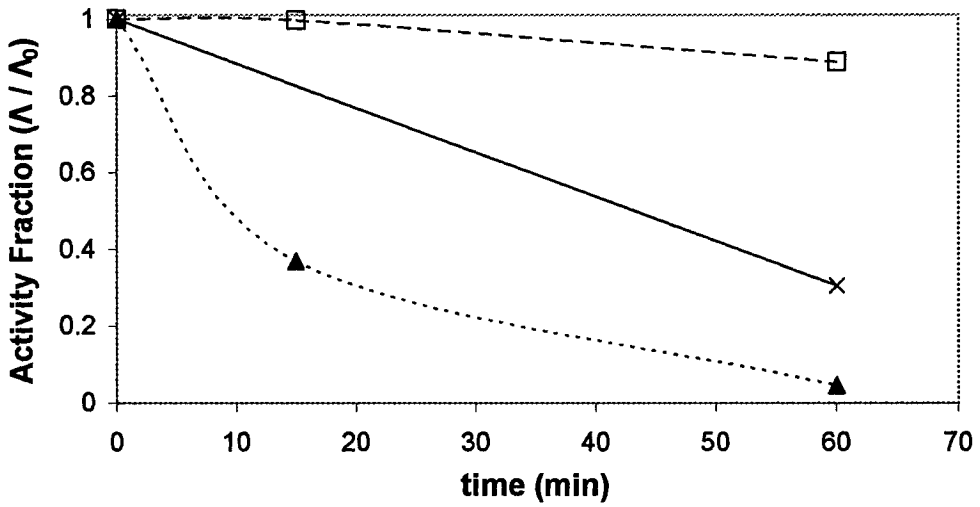


Figure 3-8: Deactivation of filtered cell free extracts (···▲···), resting cells (--□--), and centrifuged cell free extracts (—×—) 22°C. Λ stands for activity.

The caffeine degradation rates of resting cell suspensions and of cell free extracts were monitored for one hour. This was done at 22°C and also at 50°C. Three measurements were taken for cell free extracts and for resting cells throughout a one hour period, at 22°C and at 50°C, as described in section 2.6. The time when the first assay was started was defined as time zero, and the activity obtained from the first assay was used to normalize the second and third assays. Initial activities were one order of magnitude higher for resting cells than for cell free extracts at 22°C (Figure 3-6 and Table 3-3). Figure 3-8 shows the stability experiment results at 22°C. Resting cells lost 11 % activity in one hour while filtered cell free extracts lost 95 % in 1 hour, and cell free extracts prepared by centrifugation lost 70 % in 1 hour (Figure 3-8). Initial activities were very

similar for resting cells and for cell free extracts at 50°C (Figure 3-6). The stability experiment results at 50°C are shown in Figure 3-9. Resting cells lost 17 % activity in 1 hour, and cell free extracts lost 99 % in 15 minutes (Figure 3-9).

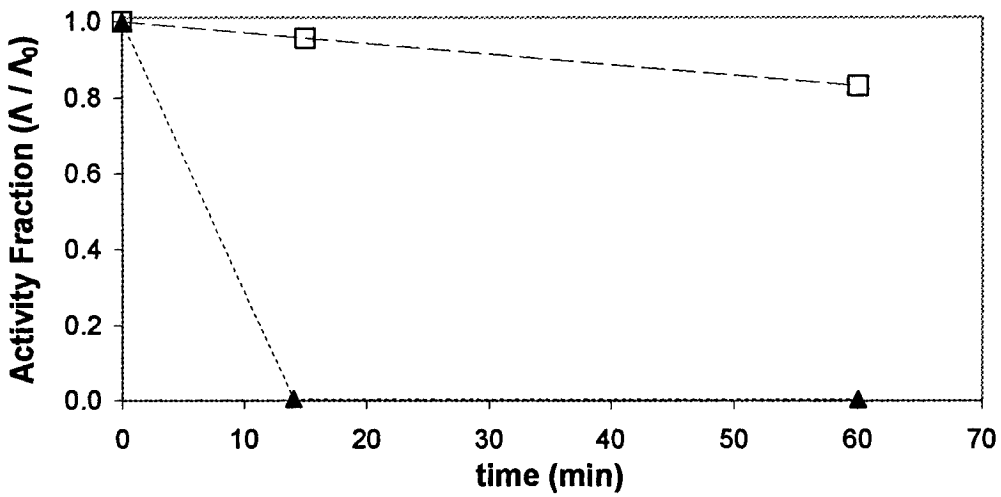


Figure 3-9: Deactivation of filtered cell free extracts (···▲···) and resting cells (--□--) at 50°C. **Λ** stands for activity.

3.4 Protein Identification by Size

SDS-PAGE was performed on *Pseudomonas putida* IF-3's cell free extracts in order to identify by size the proteins responsible for caffeine demethylation. An over expressed protein unit was found, and its molecular weight determined to be 41 kDa (Figure 3-10). The composition of the ladder used as a molecular weight standard is described in section 2.2.5.

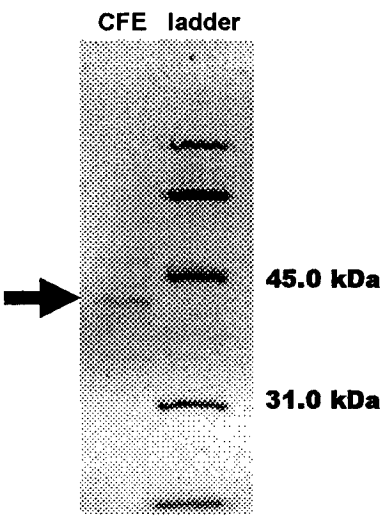


Figure 3-10: SDS-PAGE of cell free extracts (CFE). The black arrow points to the over-expressed protein unit in *P. putida* IF-3's cell free extracts. The protein's molecular weight was determined to be 41 kDa. The 45 kDa mark corresponds to ovalbumin and the 31 kDa standard corresponds to carbonic anhydrase (section 2.2.5).

4. Discussion

The objective of this study was to quantify the activity and stability of caffeine demethylases, in resting cells and cell free extracts of *Pseudomonas putida* IF-3, at different temperatures. For this purpose, assays were developed for both resting cells and cell free extracts (Section 2.5), and a method to compare caffeine degradation rates, on a weight of dry cells per volume basis, was devised (Section 3.1.3).

It was found that resting cells were able to degrade caffeine 12 times faster than cell free extracts at 22°C (Figure 3-6 and Table 3-3), yet at 50°C resting cells and cell free extracts exhibited similar caffeine degradation rates (Figure 3-6). At 22°C, resting cell caffeine degradation rates reduced by 11 % in one hour, while cell free extract caffeine degradation rates decreased by 95 % in the same period of time (Figure 3-8). At 50°C, resting cell caffeine degradation rates slowed down by 17 % in one hour, while cell free extract caffeine degradation rates were diminished by 99 % in only 15 minutes (Figure 3-9).

SDS-PAGE results (Section 3.4) suggested that cell free extracts from *P. putida* IF-3 contain an over-expressed protein of similar size to the ones previously reported for cell free extracts from other *P. putida* strains capable of degrading caffeine (ASANO et al. 1994; OGUNSEITAN 2002; SIDESO et al. 2001). However, it was surprising to find that caffeine degradation rates were 55 times higher than previously reported rates (SIDESO et al. 2001) (Table 4-1). This result may be attributed to two reasons. First, the strain of

P. putida used in this study (*P. putida* IF-3) was different to the strain used by Sideso (*P. putida* KD6) (SIDESO et al. 2001). Even though the over expressed proteins were of similar sizes in both organisms, different bacterial strains have different metabolic capabilities. Second, Sideso (SIDESO et al. 2001) used sonication to disrupt cells, while fluid shear was employed as a lysis method for this study. It is plausible that lysis by nebulization did not affect the activity of the caffeine demethylases as much as lysis by sonication.

System	Rate ($\times 10^3$ mmol caffeine / g protein · min)
Filtered Cell Free Extracts	8.30 ± 1.30
Cell Free Extracts (Sideso et al, 2001)	0.15

Table 4-1: Comparison between obtained caffeine degradation rates and literature at room temperature.

This study was designed to test the hypothesis that when compared on the same basis, resting cells of *Pseudomonas putida* IF-3 would degrade caffeine at a faster rate than the cell free extracts. Results (Figure 3-6) suggest that this is true in the 22°C to 42°C interval; however degradation rates appear to be very similar at 50°C (Figure 3-6).

Resting cell degradation rates were found to be an order of magnitude higher than cell free extract degradation rates at 22°C (Table 3-3). Resting cells were likely to have not

only their metabolic machinery intact, but also to keep an optimal environment for caffeine demethylases inside the cytosol.

After cell lysis, a 60 % reduction in caffeine degradation rates was observed in the lysate before separating the cell debris (Figure 3-7). One possible explanation for this loss is the change in chemical environment after cell lysis. Cell disruption introduces at least three possible types of reaction: 1) establishment of new macromolecular cross linkages when intercalated water molecules are withdrawn or added; 2) establishment of new solute-macromolecular interactions; 3) modification of solute potentials by precipitation formation of single solutes or by alteration of solute-solute complexes (BATEMAN and WHITE 1963). The interplay of these reactions may have induced enough structural changes in caffeine demethylases to produce less active configurations after lysis (Figure 3-7 and Table 3-4). In addition cell lysis is also known to cause immediate dilution of solutes present in the cytoplasm (BAILEY and OLLIS 1986). This sudden dilution could have affected degradation through a sudden decrease in the concentration of key ions. Previous studies suggested that changes in the concentration of divalent ions could affect demethylating rates of *P. putida* cell free extracts by as much as 53 % (HOHNLOSER et al. 1980). However, this hypothesis was not tested. The cell disruption mechanism itself may have also been responsible for the observed loss of activity after lysis. Even though little is known about cell disruption by nebulization, the atomization of the cell suspension by the BioNeb results in significant shear, possibly enough to cause enzyme deactivation (CARLSON et al. 1995).

Following cell debris removal after lysis, either by filtration or by centrifugation, a considerable reduction in caffeine degradation rates was also observed at 22°C. After filtration, caffeine degradation rates reduced by 30 %, and after centrifugation by 17 % (Figure 3-7). One possible explanation for this difference is the induced shear stress on the lysate, associated with each separation method. It was estimated that filtration could induce stresses up to five orders of magnitude higher than centrifugation (calculations not shown). Separation could have removed cofactors, which in some cases may include, apart from specific coenzymes, structural cell components such as membranes containing lipids (DIXON and WEBB 1979). It has been proposed previously that the properties of some enzymes appear to be modified through interaction with membranes within the cell; even membrane fragments could act as cofactors for some enzymes (DIXON and WEBB 1979). Moreover, separation steps could remove substances of various kinds that strictly speaking are not activity-producers but rather activity-preservers (DIXON and WEBB 1979). Indeed centrifuged cell free extracts were more stable than filtered cell free extracts. Figure 3-8 shows filtered cell free extracts lost 95 % of their initial activity in one hour, while centrifuged cell free extracts lost 70 % of their initial activity. Centrifuged cell free extracts, being more turbid than filtered cell free extracts, could have stabilized the demethylases, perhaps through hydrophobic interactions with left over membrane fragments. It is well known that membrane fragments may associate via hydrophobic bonding of the phospholipids (BROCK 1979). Lastly, the decrease in caffeine degradation rates with the course of time, of both centrifuged and filtered cell free extracts at room temperature (Figure 3-8), seems to agree with the rapid cell free

extract deactivation at room temperature reported by previous researchers (ASANO et al. 1994; SIDESO et al. 2001).

NAD turnover could have also affected the ability of cell free extracts to degrade caffeine. It has been proposed in the past that two different enzymes could act cooperatively in cell free extracts of *P. putida*: the first one catalyzes the demethylation of caffeine in the presence of NADH and O_2 to produce theobromine, water, formaldehyde and NAD^+ (Figure 1-7), while the second one oxidizes formaldehyde to formic acid, and at the same time it regenerates NAD^+ to NADH (Figure 1-8) (HOHNLOSER et al. 1980). Evidence for an enzyme that oxidizes formaldehyde in cell free extracts of *P. putida* was also found by Blecher (BLECHER and LINGENS 1977) and Asano (ASANO et al. 1994). Cell free extract experiments did not show NADH regeneration at appreciable rates, neither in the presence nor in the absence of caffeine (Figure 3-1). Based on this result, it is plausible that the cell free extracts were limited by the turnover of NAD.

It was hypothesized that temperatures near coffee drinking temperature (50°C) will cause a rapid reduction in the caffeine degradation rates of resting cells, and of cells free extracts of *Pseudomonas putida* IF-3 in buffered, aqueous solutions. The observed trend for resting cell caffeine degradation rates with respect to temperature (Figure 3-6), resembles a previous report on the behavior of *P. putida* cultures, grown on caffeine as their sole source of carbon and nitrogen. Fastest growth has been reported to be between 25°C and 30°C (BLECHER and LINGENS 1977) and optimal growth temperature 30°C

(KOIDE et al. 1996). Similarly, resting cell degradation rates reached a maximum near 30°C (Figure 3-6). The substantial drop in resting cell caffeine degradation rates from 33°C to 50°C (Figure 3-6) suggests that further increases in temperature would result in no caffeine degradation by resting cells; correspondingly, no growth has been reported at 40°C, and death of *P. putida* IF-3 has been reported to occur at 60°C (KOIDE et al. 1996). Resting cell activity-loss, with increasing temperature, was fast enough to decrease the observed caffeine degradation rates, right after preparing the cell suspension, by 77 % between 33°C and 50°C (Figure 3-6). This result supports the second working hypothesis. However, the relative decrease in caffeine degradation rates did not vary as much between 22°C and 50°C: in one hour, 11 % activity was lost at 22°C and 17 % at 50°C (Figure 3-8 and Figure 3-9). This result seems to indicate that a sudden increase in temperature brings about a rapid reduction in caffeine degradation rates. Nonetheless after that first, fast reduction in rates, activity loss is much more gradual.

Increasing temperature did not affect cell free extract caffeine degradation rates in the same manner as it affected resting cells. Caffeine degradation rates measured immediately after cell free extract preparation augmented with increasing temperature, even above the optimal growth temperature (30°C), (Figure 3-6). Similar behavior has been reported for several enzymatic systems of other strains of *Pseudomonas putida*, where key enzymes remained active up to 60°C (LEE and RHEE 1993; OSAKI and SAKIMAE 1997). Activation and deactivation of enzymes with increasing temperatures can occur simultaneously (SHULER and KARGI 1992), and from our results it could be conjectured that activation occurred at a faster rate than deactivation, possibly explaining

the rise in initial rates with increasing temperature (Figure 3-6). However, the relative deactivation rate of cell free extracts was 4 times faster at 50°C than at 22°C (Figure 3-8 and Figure 3-9), indicating that although initial activity increases with increasing temperature, stability of the enzyme is highly compromised after prolonged exposure to high temperatures.

5. Limitations of this Study

5.1 Caffeine Degradation Rates

Cell free extract, caffeine degradation rates were calculated using the fact that no caffeine degradation was possible without the presence of NADH (Section 2.5.1). Although this method proved to be reliable, the obtained rates were conservative estimates of the time it takes cell free extracts to degrade caffeine. This means that caffeine degradation could have occurred at a faster rate.

5.2 Lysis Quantification and Equivalent Dry Cell Concentration

The lysis quantification method (Section 3.1.2) was based on being able to treat cell lysis as a first order process, yet most importantly on being able to bound the first order constant to a maximum value associated with 100 % lysis (Section 3.1.2). The process of choosing the maximum value for that first order constant was empirical, meaning that after more than 90 lysis procedures with *P. putida* IF-3 suspensions, it was found that the **k**-value converged to 0.15 min^{-1}, but never reached 0.15 min^{-1}. Like any empirical method, it may be subject to bias if the number of procedures was not large enough in order to obtain a truly representative **k**-value. This limitation links to the equivalent dry cell concentration for cell free extracts (Section 3.1.3), for lysis efficiency was used to correct for unlysed cells when calculating cell free extract caffeine degradation rates.

In addition, the lysis efficiency quantification method proposed here is not only organism specific, but also lysis conditions specific. Should this method be applied to quantify lysis for other organisms or for other nebulizing conditions, new curves such as the ones in Figure 3-4 and Figure 3-5 should be produced. Also the number of lysis procedures should be sufficiently large so that the chosen maximum **k**-value is truly representative of 100 % lysis, thus avoiding introduction of bias when calculating equivalent dry cell concentration in cell free extracts.

5.3 Pure Caffeine, Aqueous, Buffered System

Since the response of enzymes vary greatly not only with pH but also with changing aqueous environment, caution should be used before drawing general conclusions from the observations in this study. Pure caffeine, buffered solutions were used for caffeine degradation experiments, thus extrapolation of the results shown here to decaffeination rates for beverages should be avoided.

6. Conclusions

- A novel method to quantify lysis efficiency was developed. It was found that the rate of lysis, by fluid shear, can be treated as a first order process. Determination of the first order constant of each lysis step allowed lysis efficiency quantification.

- Cell free extracts from *Pseudomonas putida* IF-3 were found to degrade caffeine at least 55 times faster than previously reported cell free extracts of other caffeine degrading strains of *P. putida.*

- Centrifuged cell free extracts were found to be almost three times more active that filtered cell free extracts at 22°C. In addition, the cell debris separation method appeared to influence the stability of cell free extracts.

- Between 22°C and 50°C, resting cells of *Pseudomonas putida* IF-3 were one order of magnitude faster than cell free extracts, at degrading caffeine; however, resting cell activity dropped above 40°C, and at 50°C both systems had equivalent caffeine degrading activity.

- As temperature was raised between 20°C and 50°C, cell free extract caffeine degradation rates increased, yet stability of the extracts was compromised, and activity practically disappeared in 15 minutes, at 50°C.

- Resting cells are more stable than cell free extracts; this was significantly more evident at 50°C than at 22°C. While cell free extracts lost all activity in 15 minutes at 50°C, resting cells retained 87 % of their initial activity after one hour at 50°C.

7. Suggestions for Future Work

- Cell free extract experiments should be performed at temperatures above 50°. In that way the temperature at which cell free extract activity starts diminishing could be determined. In the same manner the temperature at which resting cell activity reaches zero could be determined.

- Stability experiments should be performed for centrifuged cell free extracts at 50°C. The results would determine whether centrifuged cell free extracts are also more stable than filtered cell free extracts at 50°C. (It was shown in this work that centrifuged cell free extracts are more stable than filtered cell free extracts at 22°C.)

- Cell free extracts from *P. putida* IF-3 could be prepared using various cell disruption methods in order to assess the impact of the cell lysis method in cell free extract activity.

- Cell free extract experiments could be performed using varying concentrations of several divalent ions. Results could confirm the proposed hypothesis that changing the concentration of these ions has a considerable effect on demethylating activity (Section 4).

- Cell free extract experiments could be performed using varying concentrations of phospholipids. Results could confirm the proposed hypothesis that changing the concentration of phospholipids (membrane fragments) has a considerable effect on both demethylating activity and stability (Section 4).

- When performing cell free extract, caffeine degradation experiments, samples should be tested for formic acid production. The presence of formic acid would further support the proposed hypothesis that cell free extract, caffeine degradation rates are limited by the turnover of NAD.

- Adding known protein stabilizing agents to cell free extracts could make the difference when selecting whether resting cells or cell free extracts are more effective at degrading caffeine, remembering that at 50°C both systems are equivalently active but resting cells are considerably more stable.

- Coffee has a pH of about 5 and tea a pH of about 6, thus before proceeding to try beverage decaffeination caffeine, degradation experiments should be done at lower pH than 7.5.

8. References

ALAGAPPAN,G. and COWAN,R. 2004. Effect of Temperature and Dissolved Oxygen on the Growth Kinetics of *Pseudomonas putida* F1 Growing on Benzene and Toluene. Chemosphere **54:** 1255-1265.

ASANO,Y., KOMEDA,T., and YAMADA,H. 1993. Microbial Production of Theobromine from Caffeine. Bioscience, Biotechnology and Biochemistry **57:** 1286-1289.

ASANO,Y., KOMEDA,T., and YAMADA,H. 1994. Enzymes Involved in Theobromine Production from Caffeine by *Pseudomonas Putida* No. 352. Bioscience, Biotechnology and Biochemistry **58:** 2303-2304.

BAILEY,J. and OLLIS,D. 1986. Biochemical Engineering Fundamentals. McGraw-Hill.

BATEMAN,J. and WHITE,E. 1963. Relative Humidity and the Killing of Bacteria: the Survival of *Serratia marcescens* Dehydrated by Glycerol and Sucrose Solutions. Journal of Bacteriology **85:** 918-926.

Bio-Rad. DC Protein Assay Instruction Manual. 2002.

Bio-Rad. Mini-Protean 3 Cell: Instruction Manual. [165-3301 / 165-3302]. 2003.

BLECHER,R. and LINGENS,F. 1977. The Metabolism of Caffeine by a *Pseudomonas putida* Strain. Hoppe-Seyler's Zeitschrift Fur Physiology und Chemistry **358:** 807-81.

BROCK,T. 1979. Biology of Microorganisms. Prentice-Hall International.

CARLSON,A., SIGNS,M., LAURA,L., BOOR,R., and JEM,K. 1995. Mechanical Disruption of *Escherichia Coli* for Plasmid Recovery. Biotechnology and Bioengineering **48:** 303-315.

CASTLE,T. 1994. The Future of Decaf. Tea & Coffee Trade Journal **166:** 60-62.

DIXON,M. and WEBB,E. 1979. Enzymes. Academic Press.

FOLIN,O. and CIOCALTEAU,V. 1927. On Tyrosine and Tryptophane Determinations in Proteins. Journal of Biological Chemistry **73:** 627-649.

FRARY,C., JOHNSON,R., and WANG,M. 2005. Food Sources and Intakes of Caffeine in the Diets of Persons in the United States. Journal of the AMERICAN DIETETIC ASSOCIATION **105:** 110-113.

GAULIN,J. 2003. Selective Caffeine Removal by Microbial Consortia. Master of Science McGill University.

HAAS, G and STIEGLITZ, B. Microbiological Decaffeination of Aqueous Liquids. General Foods Corporation. [4228191]. 1980a. United States.

HAAS, G and STIEGLITZ, B. Microbiological Decaffeination of Aqueous Solutions . General Foods Corporation. 570, 286 and 570, 286[(Canadian Patent) 1086553]. 1980b. 2-4-0076b.

HARRIS,D. 1999. High Performance Liquid Chromatography. *In* Quantitative Chemical Analysis. W.H. Freeman and Company, New York. pp. 713-753.

HOHNLOSER,W., OSSWALD,B., and LINGENS,F. 1980. Enzymological Aspects of Caffeine Demethylation and Formaldehyde Oxidation by *Pseudomonas putida* C1. Hoppe-Seyler's Zeitschrift Fur Physiology und Chemistry **361:** 1763-1766.

JODYNIS-LIEBERT,J. and MATUSZEWSKA,A. 1999. Effect of toluidines and dinitrotoluenes on caffeine metabolic ratio in rat. Toxicology Letters **104:** 159-165.

KOIDE, Y, NAKANE, S, and YUTAKA, I. Caffeine Demethylate Gene-Containing DNA Fragment and Microbial Process for Producing 3-methyl-7alkylxanthine. Amano Pharmaceutical Co. [US 5550041]. 1996.

KOLAYLI,S., OCAK,M., KÜÇÜK,M., and ABBASOGLU,R. 2004. Does Caffeine Bind to Metal Ions? Food Chemistry **84:** 383-388.

LAEMMLI,U.K. 1970. Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4. Nature **227:** 680-685.

LEE,S. and RHEE,J. 1993. Production and Partial Purification of a Lipase from *Pseudomonas putida* 3SK. Enzyme and Microbial Technology **15:** 617-623.

LOWRY,O., ROSEBOROUGH,N., FARR,A., and RANDALL,R. 1951. *ProteinMeasurement with the Folin Phenol Reagent.* Journal of Biological Chemistry **193:** 265-275.

MIDDELHOVEN,W.J. 1982. Degradation of Caffeine by Immobilized Cells of *Pseudomonas putida* Strain C 3024. Applied Microbiology and Biotechnology **15:** 214-217.

MIDDLEHOVEN,W.J. 1982. Degradation of Caffeine by Immobilized Cells of *Pseudomonas putida* Strain C 3024. Applied Microbiology and Biotechnology **15:** 214-217.

OGUNSEITAN,O. 1996. Removal of caffeine in sewage by *Pseudomonas putida*: implications for water pollution index. World Journal of Microbiology and Biotechnology **12:** 251-256.

OGUNSEITAN,O. 2002. Caffeine-inducible enzyme activity in *Pseudomonas putida* ATCC 700097. World Journal of Microbiology and Biotechnology **18:** 423-428.

OSAKI,E. and SAKIMAE,A. 1997. Purification and Characterization of Recombinant Esterase from *Pseudomonas putida* MR-2068 and Its Application to the Resolution of Dimethyl Methylsuccinate. Journal of Fermentation and Bioengineering **83:** 535-539.

OSORIO, N. Action to Address the Coffee Crisis. 2003. G-8 Summit: Evian, France, International Coffee Organization.

PETERSON,G. 1979. Review of the Folin Phenol Protein Quantitation Method of Lowry, Roseborough, Farr and Randall. Analytical Biochemistry **100:** 201-220.

PETERSON,J. and GRAHAM-LORENCE,S. 1995. Bacterial P-450s. *In* Cytochrome P-450: Structure, Mechanism and Biochemistry. *Edited by* P.ORTIZ DE MONTELLANO. Plenum Press, pp. 151-180.

PORTER,T. and COON,M. 1991. Minireview: Cytochrome P-450. The Journal of Biological Chemistry **266:** 13469-13472.

REINER,J. 1969. Behavior of Enzyme Systems. Van Nostrand Reinhold Company.

SAMBROOK,J., FRITSCH,E., and MANIATIS,T. 1989. SDS-Polyacrylamide Gel Electrophoresis of Proteins. *In* Molecular Cloning: a Laboratory Manual. *Edited by* N.FORD, C.NOLAN, and M.FERGUSON. Cold Spring Habor Laboratory Press, pp. 18.47-18.59.

SHULER,M. and KARGI,F. 1992. Bioprocess Engineering. Prentice Hall.

SIDESO,O., MARVIER,A., KATERELOS,N., and GOODENOUGH,P. 2001. The Characteristics and Stabilization of a Caffeine Demethylaze Enzyme Complex. Revista de Microbiologia **36:** 693-698.

SMITH,J.M., VAN NESS,H.C., and ABBOT,M.M. 1996. Introduction to Chemical Engineering Thermodynamics. McGraw Hill.

SOLOMONS,G. 1996. Organic Chemistry. John Wiley & Sons.

TOGASAKI, R, SURZYCKI, S, and YOKOTA, H. BioNeb® Cell Disruption System Instruction Manual. 3.0. 2000. Bloomington, Indiana (USA).

WHITE,R. and COON,M. 1980. Oxygen Activation by Cytochrome P-450. Annual Reviews in Biochemistry **49:** 31-56.

WOOLFOLK,C.A. 1975. Metabolism of n-methylpurines by a *Pseudomonas putida* strain isolated by enrichment on caffeine as the sole source of carbon and nitrogen. Journal of Bacteriology **123:** 1088-1106.

WORLD HEALTH ORGANIZATION: INTERNATIONAL AGENCY FOR RESEARCH ON CANCER. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Coffee, Tea, Mate, Mehtylxanthines and Methylglyoxal. [51], 41-347. 1991.

YAMAOKA-YANO,D. and MAZZAFERA,P. 1999. Catabolism of Caffeine and Purification of a Xanthine Oxidase Responsible for Methyluric Acids Production in *Pseudomonas putida* L. Revista de Microbiologia **30:** 62-70.