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OPTIMISATION OF MEDIUM AND ITS COMPONENTS FOR EFFICIENT CAFFEINE DEGRADATION BY *BREVIBACTERIUM*

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Abstract: *Caffeine (1,3,7-trimethylxanthine), a purine alkaloid occurs in more than 60 plant species including in the seeds of coffee, cacao, cola tree and in the leaves of tea (Ashihara and Crozier, 2001).Concerned with the deleterious effects of potential chronic ingestion of caffeine on the physiological systems(higher than 150mg / day),decaffeination is highly recommended in both coffee processing and disposal of coffee spent.*

Conventional decaffeination techniques like solvent extraction or use of supercritical carbon dioxide can be expensive, toxic to the environment and non-specific. So there is a strong need for caffeine degradation by alternative routes other than conventional techniques. The potential use of microbes and their enzymes is an attractive alternative as it is cheap, easier and faster. (Mazzefera et al., 2002).The present study aims in optimizing physical parameters and media for maximum degradation of caffeine by the selected isolate Brevibacterium.

Harnessing the caffeine degrading potential of organisms growing in caffeine rich soil is of importance in developing processes for biodecaffeination and production of methylxanthine intermediates which have therapeutic value.

The present investigation mainly deals with the Optimization of media and its main components viz., effect of Carbon source and Nitrogen Source to achieve maximum biodegradation of caffeine by the selected isolate Brevibacterium isolated and maintained in our laboratory. The growth and caffeine degradation were recorded as the increase in biomass by weight and residual caffeine analysis of the samples by HPLC respectively.

Brevibacterium was grown in the optimized growth medium containing sucrose (10 g.L-1), caffeine (2 g.L-1), yeast extract (15 g.L-1), peptone (30 g.L-1) and ammonium sulphate (15 g.L-1). The temperature of the medium was set at 35^{o}C, and pH adjusted to 7.0 and an inoculum of 7 %w/v was added to the medium and incubated by shaking at 150rpm for 96 hrs. Under these conditions, 9.6 g.L-1 of biomass was accumulated after 96 hrs with a growth rate of 0.031 g.L-1.h-1. Caffeine was almost completely (99.8%) degraded within 60 hrs of incubation. High caffeine degradation rates were observed under these conditions. The caffeine degradation rate was 0.034 g.L-1.h-1. Under optimized conditions the acclimatization time for caffeine was reduced to 24hrs and around 50%.

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1. INTRODUCTION

Caffeine (1,3,7-trimethylxanthine), a purine alkaloid occurs in more than 60 plant species including in the seeds of coffee, cacao, cola tree and in the leaves of tea (Ashihara and Crozier, 2001).Concerned with the deleterious effects of potential chronic ingestion of caffeine on the physiological systems(higher than 150mg / day),decaffeination is highly recommended in both coffee processing and disposal of coffee spent.

Conventional decaffeination techniques like solvent extraction or use of supercritical carbon dioxide can be expensive, toxic to the environment and non-specific.so there is a strong need for caffeine degradation by alternative routes other than conventional techniques. The potential use of microbes and their enzymes is an attractive alternative as it is cheap, easier and faster. (Mazzefera et al., 2002).The present study aims in optimizing physical parameters and media for maximum degradation of caffeine by the selected isolate *Brevibacterium*.

2. MATERIALS AND METHODS

2.1Chemicals

Caffeine, Theobromine, Paraxanthine and Methyl xanthines were purchased from Sigma Chemicals, St. Louis, USA. Nutrient broth, nutrient agar, citrate agar, peptone, yeast extract, and other chemicals for identification of bacterial isolates were procured from Hi Media labs, Mumbai, India. HPLC grade acetonitrile and methanol were procured from Merck, Germany. All other chemicals were of the highest purity and were procured from standard sources.

The bacterial isolate *Brevibacterium* from our laboratory was found to amplify and withstand in high caffeine concentration (8g/L)and was taken into this investigation.

2.2 Growth of cells and induction for caffeine degradation

A loop full of actively growing culture of the isolate from the culture slant was transferred to 100ml of nutrient broth containing 0.3 g.L-1 caffeine and incubated at 30^{o}C in an orbital shaker for 24 hrs. A 5% v/v of the 24 hrs grown pre inoculum was transferred to 100ml of nutrient broth containing 0.3 g.L-1 caffeine and grown under the same conditions. Samples were drawn at known intervals of time for the measurement of cell growth. Biomass

accumulated after 24 hours was harvested by centrifugation in a bench top centrifuge (Kubota, Japan) at 16,000g for 20 min at 0-4^{0}C to form a pellet.

The biomass pellet was aseptically transferred into a 500ml flask containing 100ml of CLM containing 1g.L-1 caffeine and incubated at 30^{o}C on the orbital shaker for a period of 48hrs for inducing the cells to degrade caffeine. These induced cells were harvested by centrifugation as before. The cells were washed several times to remove caffeine. 10 grams of these induced cells were suspended in 100 ml of phosphate buffer containing caffeine, which were used for caffeine degradation experiments.

2.3 Optimization of laboratory conditions for the growth of caffeine degrading bacteria

2.3.1Screening of media

The following 4 different media (Table 1) were initially screened for the growth and caffeine degrading efficiency by the isolates. The composition of media is given below (g.L-1). The pH of all the media was adjusted to 7.0, and sterilized at 121°C for 15 min at 15 PSI, cooled and inoculated with a loop full of actively growing culture of *Brevibacterium*.

Table 1.Composition of media screened for caffeine degradation and growth of *Brevibacterium spp.* Media Composition (g.L-1), pH 7.0

CM1. Nutrient Broth		**CM 2. Defined Medium**	
Peptones	5.0	Sucrose	10.0
Yeast extract	3.0	K2HPO4	2.5
Sodium chloride	6.0	KH2PO4	2.5
Beef Extract	15.0	(NH4)2HPO4	1.0
		MgSO4 7H2O	0.20
		FeSO4 7H2O	0.01
		MnSO4 H2O	0.007
CM 3. Glucose- NH4SO4 Medium :		**CM 4. Complex organic media**	
Glucose	50.0	Glucose	25.0
Na2HPO4.12 H2O	16.0	Yeast extract	3.0
KH2PO4	2.96	(NH4)2SO4	2.0
NH4SO4	5.0	CaCO3	2.0
MgSO4	1.0	NaCl	2.0
FeSO4	0.0002	KH2PO4	2.0

2.3.2 Effect of carbon source on caffeine degrading B*revibacterium* Sp

Carbon sources are required for the basic metabolic activities, growth and modulating the organisms' metabolic activities either enabling them to use a substrate such as caffeine or regulating the uptake of the substrate itself. Fructose, sucrose, glucose, inulin, raffinose and starch were checked for their suitability as the carbon sources for growth as well as caffeine

degradation by the organism. The carbon sources were added individually at 10g.L-1 concentrations into Caffeine liquid medium (CLM) and it served as the basal medium for the studies.

2.3.3Effect of nitrogen source on caffeine degrading *Brevibacterium*

The various organic nitrogen sources tried were yeast extract, tryptone, Peptone and beef extract. The inorganic nitrogen sources tried were ammonium nitrate, ammonium sulphate and urea. The nitrogen sources were added individually at 10 g.L-1 concentrations into caffeine liquid medium, which served as the basal medium for the studies.

2.4 Biomass determination:

The cell pellets after centrifugation of the culture samples were washed twice with deionized water and O.D 600 nm was measured. For cell dry weight (O.D600 nm of 0.5 corresponds to 0.379 g dry weight /100ml according to standard curve).

2.5 Estimation of methylxanthines by high performance liquid Chromatography (HPLC):

HPLC analysis of caffeine was performed in a Shimadzu LC 10 A- HPLC

System, and the Methylxanthine compounds were separated on a C18 ODS-Luna column under isocratic conditions with 15 % acetonitrile in water at a flow rate of 1.0 ml/min. Compounds eluting from the column were detected at 273 nm, and the peak areas were compared with those obtained with standards of known concentration

3. RESULT AND DISCUSSION

3.1 Screening of media:

Microorganisms have specific growth requirements in terms of substrates for growth and utilization of different media components. An initial screening in different complex media was carried out to check for the specific nutritional requirements of *Brevibacterium.*. In Growth medium containing sucrose (CM1), maximum biomass accumulation of 2.137 g.L-1 and maximum caffeine concentration (84.15%) was observed. Caffeine media (CM1) also supported good growth of the organism and the biomass accumulated in these media was 1.611.

Table 2 showing Caffeine degradation by *Brevibacterium* and its biomass concentration grown in different media

MEDIA	BIOMASS CONCENTRATION (g/L)	CAFFEINE DEGRADATION (%)

CM1	1.611	67.23
CM2	2.137	84.15
CM3	0.789	54.23
CM4	0.853	49.29

3.2 Effect of carbon source on growth and caffeine degradation by *Brevibacterium*:

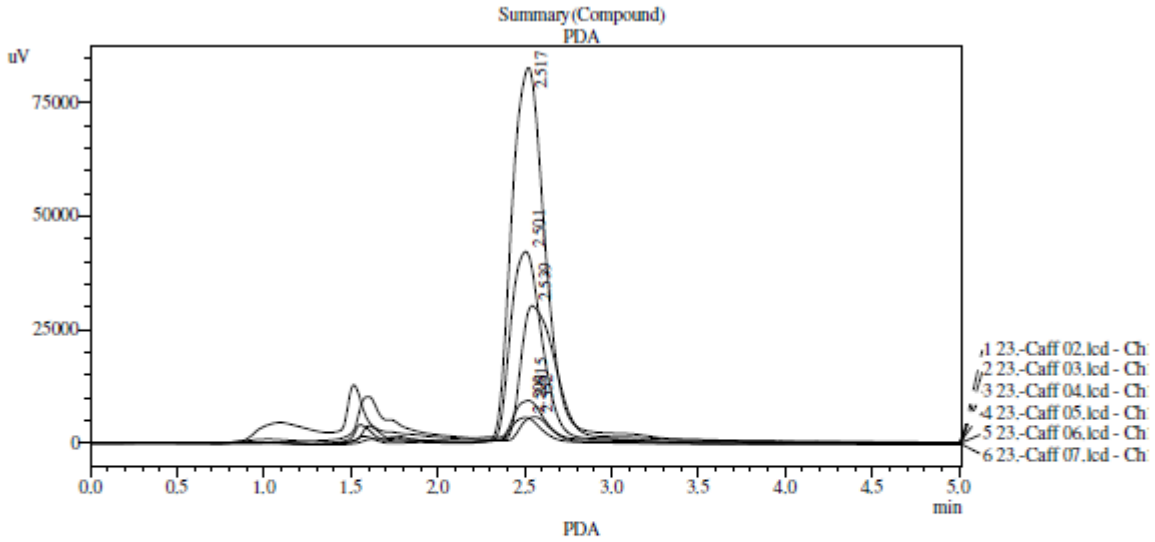
Different carbon sources exert different activities on the degradation of a substrate like caffeine. Of all the carbon sources tested Sucrose was found to be the best both in terms of growth and caffeine degradation. In the presence of Sucrose as the sole source of carbon, 4.082g.L-1 of biomass was accumulated in the medium after 72 hrs of growth and 97% of the initial caffeine was degraded within 72 hrs.

Glucose and fructose although were simpler substrates showed a lower growth and caffeine degradation. Biomass accumulation was only 1.056 and 0.169 g.L-1 for glucose and fructose respectively. Caffeine degradation was also slow with on 48 and 39 % caffeine degraded in 72 hrs by glucose and fructose respectively. Other sugars were found to have an inhibitory effect both on the growth and caffeine degradation by *Brevibacterium*.

Table 3 Effect of carbon source on growth and caffeine degradation by Brevibacterium

CARBON SOURCE	BIOMASS CONCENTRATION (g/L)	CAFFEINE DEGRADATION (%)
Fructose	0.169	39.02
Glucose	1.056	48.02
Sucrose	4.082	97.87
Inulin	1.116	8.93
Raffinose	0.052	5.02
Starch	0.179	4.13

Inulin, Raffinose and Starch are complex sugars and the organism might not be having the necessary enzymes required to utilize these substrates. Sucrose being a disaccharide is metabolized relatively slowly leading to a slow release of monosaccharides providing energy for growth as well as enabling the expression of enzymes involved in the degradation of caffeine.



<< PDA >>
ID#1 Compound Name: RT2.539

Title	Sample Name	Sample ID	Ret. Time	Area	Height	Caffeine
D:\SPINCO\data files\PDA\Project\projec	Fructose	Biomass conc 0.169	2.539	397817	29251	
D:\SPINCO\data files\PDA\Project\projec	Glucose	Biomass conc 1.056	2.501	555636	42229	
D:\SPINCO\data files\PDA\Project\projec	Sucrose	Biomass conc 4.082	2.517	1131440	82272	
D:\SPINCO\data files\PDA\Project\projec	Inulin	Biomass conc 1.116	2.515	79260	8076	
D:\SPINCO\data files\PDA\Project\projec	Raffinose	Biomass conc 0.052	2.552	65298	5514	
D:\SPINCO\data files\PDA\Project\projec	Starch	Biomass conc 0.179	2.500	66539	5469	
Average			2.521	382665	28802	

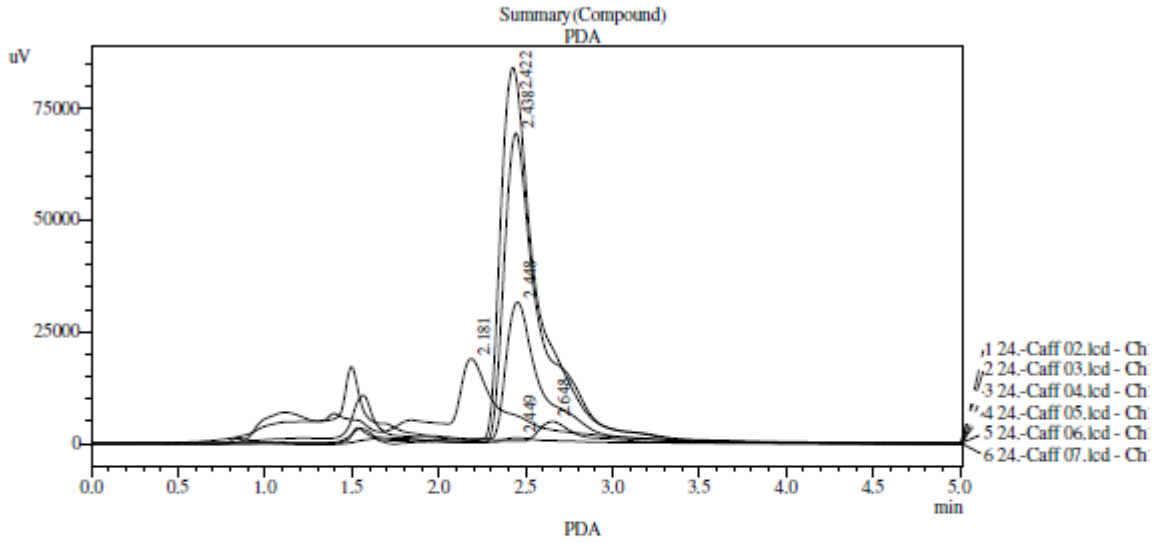
3.3Effect of nitrogen source on growth and caffeine degradation by Brevibacterium sp:

Nitrogen is a key component of the proteins and also is important for the growth of the organism. Nitrogen sources exert a significant effect on the caffeine degradation by microorganisms (Blecher, 1976; Roussos et.al, 1994; Hakil, et.al., 1999; Sánchez et.al 2004) have reported that nitrogen source and concentration play an important role in the degradation of caffeine by bacteria and fungi. Figure 3 represents the effect exerted by different nitrogen sources on growth and caffeine degradation by Brevibacterium. Maximum caffeine degradation (98.9 %) and biomass accumulation (5.923 g.L-1) was observed in medium containing yeast extract as the external carbon source. Peptone and ammonium sulphate also supported growth (3.056 and 4.512 g.L-1 respectively), whereas growth was low in tryptone, urea and ammonium nitrate (0.191, 0.164 and 0.156 g.L-1 respectively). Bacterial growth was low in media containing tryptone, and the same effect was observed in our earlier experiments too (CM5) (Fig.6.3.1). Tryptone also exerted an inhibitory effect on the caffeine degradation by the isolate and only 25% of the initial caffeine was degraded after 96 hrs of growth.

Table3 Effect of nitrogen source on growth and caffeine degradation by Brevibacterium sp:

NITROGEN SOURCE	BIOMASS CONCENTRATION (g/L)	CAFFEINE DEGRADATION (%)
Peptone	3.056	40.83
yeast extract	5.923	98.9
Tryptone	0.191	8.98
ammonium sulpahate	4.521	87.64
ammonium nitrate	0.156	5.67
Urea	0.164	20.34

Urea also did not support growth of the organism and the caffeine degradation was only 20.4% after 96 hrs of growth. This is because urea is a product of the decaffeination pathway and is at the end of the pathway. Peptone, yeast extract and ammonium sulphate were found to be the best nitrogen sources and were used for further studies on degradation of caffeine by Brevibacterium.(table 3)



<< PDA >>
ID#1 Compound Name: RT2.448

Title	Sample Name	Sample ID	Ret. Time	Area	Height	Caffeine
D:\SPINCO\data files\PDA\Project\projec	Peptone	Biomass conc 3.056	2.448	422620	30446	
D:\SPINCO\data files\PDA\Project\projec	Yeast Extract	Biomass conc 5.923	2.422	1244181	83430	
D:\SPINCO\data files\PDA\Project\projec	Tryptone	Biomass conc 0.191	2.449	2454	574	
D:\SPINCO\data files\PDA\Project\projec	Ammonium Sulp	Biomass conc 4.521	2.438	1022959	69008	
D:\SPINCO\data files\PDA\Project\projec	Ammonium Nitr	Biomass conc 0.156	2.648	78028	4652	
D:\SPINCO\data files\PDA\Project\projec	Urea	Biomass conc 0.164	2.181	171572	14923	
Average			2.431	490302	33839	

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