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OPTIMISATION OF PHYSICAL PARAMETERS FOR MAXIMAL BIODECAFFEINATION WITH *BREVIBACTERIUM*

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ABSTRACT

Brevibacterium was identified as potential caffeine degrading microbe and is taken to be tested for optimisation of physical parameters viz., pH, temperature, incubation time and inoculation volume. The study aimed to achieve maximum biodegradation of caffeine. The optimal conditions were found to be 7,37^{o}c,80 Hours and 5ml respectively.

KEY WORDS: Caffeine, *Brevibacterium*, optimisation,HPLC.

1. Introduction

Caffeine (1, 3, 7-trimethylxanthine), a central nervous system stimulant, is arguably the most frequently ingested pharmacologically active substance in the world. Occurring naturally in more than 60 plants, including coffee beans, tea leaves, cola nuts, and cocoa pods, Caffeine has been part of innumerable cultures for centuries (NAP Caffeine in Food and Dietary Supplements 2013) Concerned with the deleterious effects of potential chronic ingestion of caffeine on the physiological systems (higher than 150mg / day) and environmental contamination of caffeine containing products disposed from the coffee industries, decaffeination is highly recommended in both coffee processing and disposal of coffee spent.

Caffeine stimulates the central nervous system and can produce a variety of effects elsewhere in the body. The symptoms of a caffeine overdose ("caffeinism") will vary, according to individual differences and the amount consumed. Doses ranging from 250 to 750 mg (2 to 7 cups of coffee) can produce restlessness, nausea, headache, tense muscles, sleep disturbances, and irregular heartbeats (Tarnopolsky, 1994). Doses of over 750 mg (7 cups of coffee) can produce a reaction similar to an anxiety attack, including delirium,

ringing ears, and light flashes. These amounts of caffeine may come from a single dose or from multiple doses at short intervals (Shirlow and Mathers, 1985).

Conventional decaffeination techniques (Dixon and Johnston, 1997) 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). 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 study aims in optimizing physical parameters and media for maximum degradation of caffeine by the selected isolate *Brevibacterium*, isolated and maintained in our laboratory. The physical parameters viz., effect of Carbon source, Nitrogen Source, pH, Temperature, caffeine concentration, inoculum volume and incubation time to achieve maximum biodegradation of caffeine and growth of *Brevibacterium* were also taken into consideration

2. MATERIALS AND METHODS

2.1 Isolation of bacteria

Isolation of bacteria was carried out by spread plate method and pure cultures were obtained by streak plate method. Pure cultures were maintained on nutrient agar medium at 4ºC and were sub-cultured at an interval of every 2 week.

2.2 Bacterial Identification

Various morphological, physiological and biochemical tests were performed to identify the bacteria with reference to Bergey's Manual of Systemic Bacteriology.

2.3Amplification of the caffeine tolerant bacteria

Solid screening medium (SSM) for isolating the caffeine-tolerant bacteria was prepared by mixing the mineral solution with caffeine (2.5 g/ L) and agar (1.5%) and autoclaved at 121°C for 10 min. Solid purifying medium (SPM) was also prepared as SSM except different concentrations of caffeine (1 to 10 g/L) was supplemented. Liquid

amplifying medium (LAM) was obtained after addition of caffeine (0.5 g /L) and sucrose/glucose (5.0 g /L) in the mineral solution and disinfection.

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

2.4.1 Effect of pH on Caffeine degradation by *Brevibacterium*

pH plays an important role on the growth and activity of different enzymes required for caffeine degradation. The optimization studies were carried out with respect to the maximum rate of caffeine degradation where biomass growth and caffeine utilization were maximum. 100 ml of the CLM was adjusted to pH in the range of 4.0-9.0 in 500ml Erlenmeyer flasks was inoculated with a loop full of actively growing culture and incubated at 30^{o}C for 96 hrs on an incubator shaker at 150 rpm

2.4.2. Effect of temperature on caffeine degradation by *Brevibacterium*

Temperature is an important parameter for achieving efficient biodecaffeination and has a drastic effect on the activities of the enzymes involved in biodecaffeination. 100 ml of the CLM was adjusted to pH 7.0, was inoculated with a loop full of actively growing culture and incubated at different temperatures in the range of 20-60°C for 96 hrs on an incubator shaker.

2.4.3. Effect of inoculum volume on caffeine degradation by *Brevibacterium*

Initial Inoculum volume gives a measure of the number of cells at the start of the biodecaffeination process in the liquid culture. An optimum initial cell concentration is essential for overcoming the initial lag phase by the organism in CLM. Therefore a 24 hrs old inoculum was prepared and inoculated into fresh CLM flask in the range of 1-10 % v/v, incubated at 30±2 °C for 96 hours.

2.4.4. Effect of incubation time on caffeine degradation by *Brevibacterium*

CLM was taken in 500ml Erlenmeyer flasks was inoculated with a loop full of actively growing culture and incubated at 30oC for 96 hrs on an incubator shaker at 150 rpm. Samples were collected at different intervals of time 12, 24, 36, 48, 60 and 72 hrs.

2.5Sample Analysis

The media was sterilized and a loopful of actively growing culture was inoculated into the medium and incubated on a shaker at 150rpm at a temperature of 30± 2^{o}C for 96 hours. All the above processed samples were drawn at 12 hours intervals and the growth was recorded as an increase in the biomass by weight. Caffeine degradation was followed by HPLC analysis of the residual caffeine present in the medium

2.5.1 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).

2.5.2 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

EFFECT OF pH ON THE CAFFEINE DEGRADING BREVIBACTERIUM			EFFECT OF TEMPERATURE ON THE CAFFEINE DEGRADING BREVIBACTERIUM		
pH	BIOMASS CONCENTRATION (g/L)	CAFFEINE DEGRADATION (%)	TEMPERATURE (^{O}C)	BIOMASS CONCENTRATION (g/L)	CAFFEINE DEGRADATION (%)
4	0.589	6.85	20	2.53	73.42
5	0.346	8.93	25	2.432	70.86
6	0.785	48.23	30	3.675	95.26
7	4.823	90.67	35	4.532	97.32
8	1.089	64.89	40	4.843	95.43
9	1.164	30.26	45	3.892	82.46

EFFECT OF INOCULUM VOLUME ON THE CAFFEINE DEGRADING BREVIBACTERIUM			**EFFECT OF INCUBATION TIME ON THE CAFFEINE DEGRADING BREVIBACTERIUM**		
INOCULUM VOLUME (mL)	BIOMASS CONCENTRATION (g/L)	CAFFEINE DEGRADATION (%)	TIME (Hrs)	BIOMASS CONCENTRATION (g/L)	CAFFEINE DEGRADATION (%)
1	0.601	54.24	12	2.653	80.07
2	0.178	63.89	24	4.678	95.22
3	0.189	81.02	36	4.532	98.34
5	2.673	89.43	48	4.859	97.23
7	4.843	70.46	72	3.892	85.67
10	3.689	63.89	80	0.089	93.45
			100	1.034	76.24

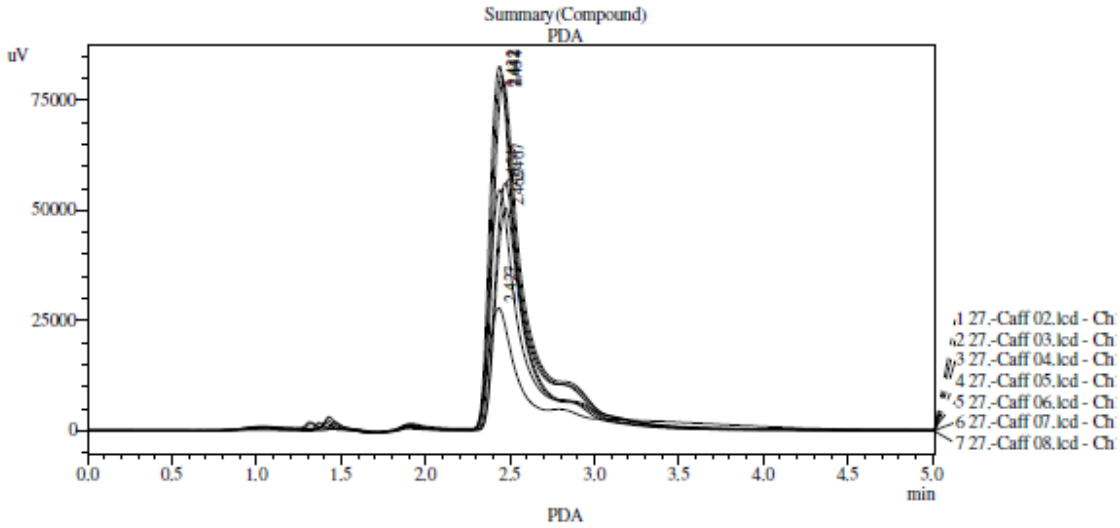
3.1 Effect of pH on growth and caffeine degradation by *Brevibacterium* :

pH plays an important role on the growth and activity of different enzymes required for caffeine degradation. Figure 6.3.4 represents the effect of pH on growth and caffeine degradation. Less than 10% of the initial caffeine was degraded by the isolate at pH 4.0 and 5.0. The efficiency of caffeine degradation increased (90.67%) as the pH of the medium was increased till 7.Highly acidic or alkaline pH was found to inhibit the growth of the organisms as well as caffeine degradation indicating that the optimum pH for the enzymes involved in caffeine degradation is near 7.0.

3.2. Effect of Temperature on growth and caffeine degradation by *Brevibacterium*:

Temperature is an important parameter for achieving an efficient Bio decaffeination and has a drastic effect on the activities of the enzymes involved in Bio decaffeination. It can be observed that maximum caffeine degradation (96.7%) was achieved when the organism was grown at 37°C and the biomass concentration (5.012 g/L) was also high at this temperature. Good Growth was observed at 25^{o}C (2.432 g.L-1) and the organism also degrades around 70% of the initial caffeine at 25^{o}C. Caffeine degradation as well as biomass accumulation was high till the temperature of the medium was increased upto 37^{o}C (4-5 g.L-1 biomass and 95-97% Caffeine degradation).

Increase in the temperature above 40^{o}C led to decrease in the growth as well as caffeine degradation by the isolate. These results indicate that the organism is a mesophile and thrives well at 35-40^{o}C with maximum efficiency of caffeine degradation. Interestingly the organism degraded around 82.46% of caffeine at 60^{0}c.

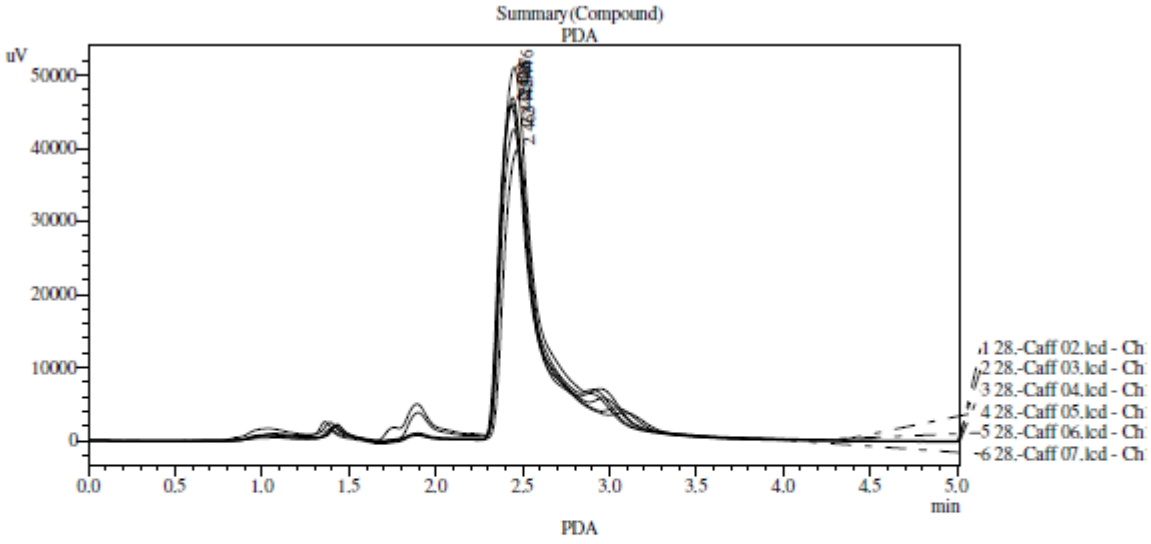


<< PDA >>
ID#1 Compound Name: caffeine

Title	Sample Name	Sample ID	Ret. Time	Area	Height	Caffeine
D:\SPINCO\data files\PDA\Project\projec	Temp 20 deg	Biomass conc 2.53	2.467	734614	55691	
D:\SPINCO\data files\PDA\Project\projec	Temp 25 deg	Biomass conc 2.432	2.434	728097	54390	
D:\SPINCO\data files\PDA\Project\projec	Temp 30 deg	Biomass conc 3.675	2.442	1057662	80001	
D:\SPINCO\data files\PDA\Project\projec	temp 35 deg	Biomass conc 4.532	2.432	1058673	81795	
D:\SPINCO\data files\PDA\Project\projec	Temp 40 deg	Biomass conc 4.843	2.454	1051252	78810	
D:\SPINCO\data files\PDA\Project\projec	temp 45 deg	Biomass conc 0.145	2.469	674334	50124	
D:\SPINCO\data files\PDA\Project\projec	Temp 60 deg	Biomass conc 0.089	2.427	368101	27149	
Average			2.447	810390	61137	

3.3. Effect of Inoculum Volume on growth and caffeine degradation by *Brevibacterium*

The optimum inoculum volume was found to be 5% inoculum volumes above this did not increase the caffeine degradation ability. There was a slight decrease in the degradation probably owing to the death of the cells. At an initial inoculum level of 1 % w/v the biomass accumulated was 0.601 g.L-1 and 54% of the initial caffeine was degraded within 96 hrs. Caffeine degradation efficiency increased with an increase in the initial inoculum volume and 89 % caffeine was degraded within 96 hrs. At inoculum volumes above 5% decrease in growth and caffeine degradation was observed. Increase in the inoculum volume will lead to increase in the density of cells in the medium. Bacterium follow the growth phase in the liquid medium and the lag, log and nutrient depletion phases will affect the metabolism of the bacteria and thus its degradation effects.

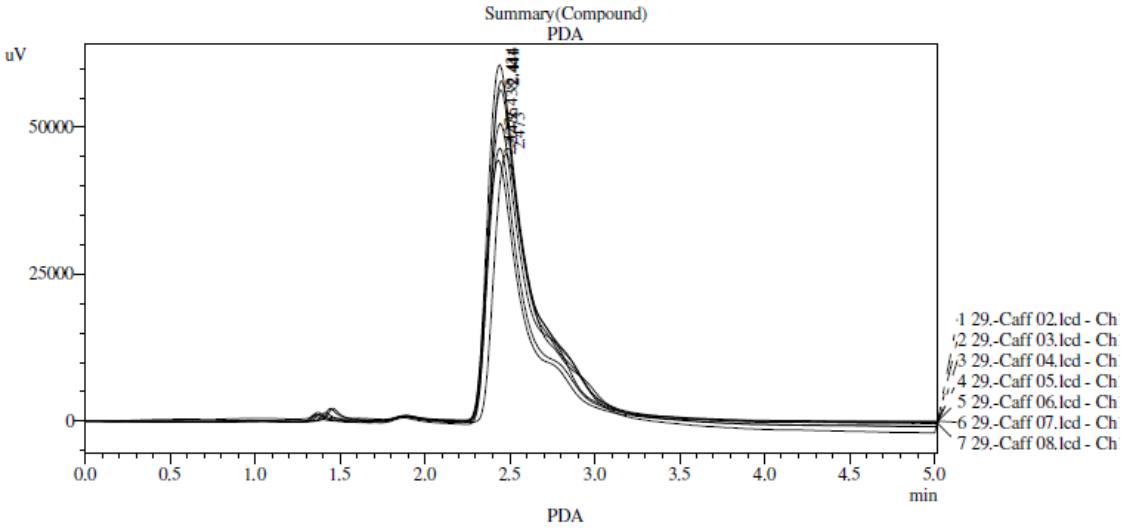


<< PDA >>
ID#1 Compound Name: caffeine

Title	Sample Name	Sample ID	Ret. Time	Area	Height	Caffeine
D:\SPINCO\data files\PDA\Project\projec	Inoculum volum	Biomass conc 0.601	2.462	590091	39236	
D:\SPINCO\data files\PDA\Project\projec	Inoculum volum	Biomass conc 0.178	2.437	687731	46574	
D:\SPINCO\data files\PDA\Project\projec	Inoculum volum	Biomass conc 0.189	2.444	589839	40967	
D:\SPINCO\data files\PDA\Project\projec	Inoculum volum	Biomass conc 2.673	2.446	731634	49804	
D:\SPINCO\data files\PDA\Project\projec	Inoculum volum	Biomass conc 4.843	2.435	704591	45766	
D:\SPINCO\data files\PDA\Project\projec	Inoculum volum	Biomass conc 3.689	2.428	679357	45391	
Average			2.442	663874	44623	

3.4.Effect of Incubation time on growth and caffeine degradation by *Brevibacterium* :

`Incubation time is an important parameter for achieving an efficient Biodecaffeination and has a drastic effect on the activities of the enzymes involved in Biodecaffeination. From Figure 6.3.8 it can be observed that maximum caffeine degradation (98%) was achieved when the organism was grown at 37° for 36 hours. Interestingly the caffeine degradation was always high till72 hours of incubation and ranged between 80 – 98%.



Conclusion

The isolate *Brevibacterium MTCC 10313* being reported is an efficient caffeine degrader, which may be useful in the development of an environmental friendly biodecaffeination process. Caffeine degradation efficiency was found to be greatly influenced by incubation time, temperature, pH, volume culture, inoculum sizes and the addition of carbon and nitrogen sources.

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