WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 7.523
Volume 6, Issue 10, 1497-1506. Research Article ISSN 2277– 7105

UTILIZATION OF BUTTERMILK PROCESSING WASTE FOR CEPHALOSPORIN C PRODUCTION BY A. CHRYSOGENUM NCIM 1069

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Article Received on
21 July 2017,
Revised on 11 August 2017,
Accepted on 01 Sep. 2017
DOI: 10.20959/wjpr201710-9480

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ABSTRACT

This study concerns the potential of dairy waste like buttermilk processing waste for Cephalosporin C production. The production, isolation and purification of Cephalosporin C by *A. chrysogenum* NCIM 1069 strain were studied. Assessment in the production of Cephalosporin C by manipulation of the dairy waste by medium formulation was used to arbitrate the strain performance. The buttermilk whey was supplemented with lactose and different organic and nitrogen sources and fermented at 30°C with agitation speed of 200 rpm for 168 hrs. The effect of different nitrogen source on Cephalosporin production was found to be insignificant. The highest Cephalosporin C (900µg/ml) was obtained from buttermilk supplemented with lactose, methionine and ammonium sulphate in cultures grown at 30°C after a period of 168 hrs. After a preliminary purification from medium using activated charcoal column, the bioassay using *A. fecalis* revealed presence and concentration of Cephalosporin. Further confirmation of the Cephalosporin was carried out by performing HPLC. This study is first report on utilization of dairy waste like buttermilk processing waste to effectively produce Cephalosporin.

KEYWORDS: buttermilk waste, cephalosporin, methionine, antimicrobial activity.

INTRODUCTION

Cephalosporin C like penicillin is an important secondary metabolite and has been the line of treatment for infections caused by bacteria on the skin, in respiratory and urinary tract (Dasari *et al.*, 2009). Although Cephalosporin C is a low potency antibiotic, more potent

semisynthetic derivatives like cephalothin, cephaloridine and cephaloglycin can be produced by modification of cephalosporin nucleus. At an industrial scale the filamentous fungus *Acremonium chrysogenum* is one of the producers of Cephalosporin C (Luoa *et al* 2013). Studies on utilization of *Streptomyces clavulingenus* (Jermini and Demain 1989, Antonio *et al* 2012, Luo *et al* 2013) and *P. persicinsis* P10 (Gohar *et al* 2013) have also been reported for production of Cephalosporin C. In past, the large scale production of Cephalosporin C has been performed using either the conventional batch process (Gohar *et al* 2013), fed batch process (Srivastava *et al* 2006) and solid state fermentation (Aharonowitz and Demain 1979, Ellaiah *et al* 2002, Tabaraie *et al* 2012, Raju *et al* 2012). Reports also suggest the comparison of free and immobilized cells for production of Cephalosporin C. Studies on nutritional requirement of these organism have been determined by using chemically defined medium (Chakraborty 2013, Gohar *et al* 2013). The attempt to increase in the yield of Cephalosporin C by either isolation of new / mutant strain or by optimization of different medium constituent are also reported (Antonio *et al* 2012). Dasari and coworkers (2009) build a model to describe the effect of moisture content, concentrations of glucose, ammonium nitrate and methionine on the yield of Cephalosporin C from *A. chrysogenum* under solid state fermentation by using artificial neural network and response surface methodology. Increase in the production of Cephalosporin C by manipulation of the medium formulation (Antonio *et al* 2012) was used to arbitrate the strain performance.

This study was intended to optimize the production of Cephalosporin C by using dairy waste like buttermilk processing waste. These dairy waste are not only freely and easily available but in a country like India where milk and milk products constituent staple part of the diet, a lot of dairy waste generated can be utilized as a cheap substrate for production of Cephalosporin C. Utilization of this dairy waste toward production of Cephalosporin C will also decrease the BOD of waste / effluent water from the dairy industry. Further buttermilk processing waste have good source of carbon like lactose and protein like casein which can be utilized by microorganism (Gupta and Prakash 2017). The waste may also contains several unique components like immunoglobulins, lactoferrin, lactoperoxidase, glycomacropeptide and sphingolipids that possess some important antimicrobial and antiviral properties. This study is first such attempt to envisage the effect of other nitrogen sources as additives for optimizing the yield of Cephalosporin C and keeping low raw material cost by utilizing buttermilk processing waste.

MATERIALS AND METHODS

Organisms used and preparation of inoculum: The fungal strain *A. chrysogenum* NCIM 1069 and the test organism *Alcaligenes faecalis* NCIM 2015 was procured from NCL, Pune, India and incubated on Potato Dextrose Agar (PDA) at 30°C. The cultures were tested for purity by microscopic and macroscopic examination and glycerol stock culture was maintained at-80°C. A seven day old culture of *A. chrysogenum* on PDA slant was suspended in sterile D/W and 10 % inoculum was used for seeding the buttermilk waste.

Culture medium and conditions: The buttermilk processing waste was kindly supplied by Rajarambapu Patil Sahakaris Dudh Utpadak Prakriya Sangh Ltd, Krishna Milk suppliers, Navi Mumbai. Physical analysis of the buttermilk waste was carried for determining the total suspended and dissolved solids (TDS and TSS) as per protocol of APHA (1998). Reducing sugar, Protein and nitrogen content of the dairy waste were determined by DNSA, biuret (Nowonty 1979) and Kjeldahl method following the procedure elaborated in Lynch and Barbano (1999). The buttermilk effluent was filtered using one layer of sterile Whatman's filter paper under vacuum conditions and autoclaved. The autoclaved, filtered buttermilk processing waste medium was assayed for microbial presence on nutrient agar and Sabouraud's agar plates for 48 hrs before proceeding for Cephalosporin production.

A total of four medium combinations were used for maximizing the Cephalosporin C production. First medium (BMP) was supplemented with only 3% lactose. The remaining three combinations of medium was supplemented with 3% lactose and 0.75% of methionine (BMLM), while the third medium was additionally supplemented with 0.1% cysteine (BMLMC) and the fourth medium with 0.4% ammonium sulphate (BMLMA). The medium was inoculated with *A. chrysogenum* and incubated at 30°C at 200 rpm for 7 days.

Extraction and Purification of Cephalosporin C: The fermentation broth was collected from each medium at the end of 4^{th}, 5^{th}, 6^{th} and 7^{th} day of incubation and centrifuged at 5000 rpm for 10 min to separate the mycelium. The fermentation broth was loaded on pre-buffered activated charcoal column (24 hrs) at pH of 6 to 6.5 followed by elution of adsorbed material from the column using a solution of 5% butanol in 0.01 N NaOH. The active charcoal so employed is treated with a phosphate buffer solution of pH 6 to 6.5 before use in order to avoid undesired changes of pH during the process of adsorption (Wildfeuer 1985). The fermentation broth eluents were collected as 1 ml fraction (a total of 5 fractions) and kept in laminar air flow to vaporize the butanol and further dried under sterile condition at room

temperature. The dried content was then dissolved in 500µl of sterile MilliQ water and stored at 4°C for further analysis.

Estimation of Cephalosporin C: The amount of Cephalosporin C was estimated spectrophotometrically at 260 nm using Cefuroxime Axetil as a standard. Cefuroxime Axetil was dissolved with MilliQ water to give a standard stock solution of 1000 µg/ml (Rimakumari *et al* 2016). The concentration of Cephalosporin C in the fractions collected from different medium and at different period of incubation was estimated against a standard graph plotted with 1000, 500, 250, 125, 62.5 µg/ml solution of Cefuroxime Axetil.

The fractions showing good amount of Cephalosporin C was further confirmed by HPLC on Waters HPLC system with a stationary phase in a C-18 prepacked column and a mobile phase of 0.5% acetic acid in acetonitrile mixed with water (at 15: 85 V/V). The fractions and standard Cephalosporin C at 1mg/ml were dissolved in 0.5% acetic acid in acetonitrile at pH 3.6. The samples were diluted to 20 µg/µl and injected with a flow rate of 2ml/min at 2765psi. The presence of Cephalosporin C was performed at 254 nm and the graph was analyzed using the HPLC chromatogram analyzing software.

Antimicrobial Activity Test of the Cephalosporin C: The fractions showing presence of Cephalosporin C both by spectrophotometerically and by HPLC were further checked for antibacterial activity by a well diffusion bioassay using log phase suspension of *Alcaligenes faecalis* NCIM 2015 (Bond *et al* 1962) with an O. D of 0.5 at 570 nm.

RESULT AND DISCUSSION

Raw milk is generally processed in the dairy industry to products such as consumer milk, butter, cheese, yogurt, condensed milk, dried milk (milk powder) and ice cream. The well-known processes such as chilling, pasteurization, fermentation, addition of sweeteners, fortification and homogenization can typical lead to increased production of by-products such as buttermilk, whey and their derivatives (World Bank. 1998). Routine procedure in a dairy industry perform procedures like cleaning, sanitization, heating, cooling, floor washing which requires huge amount of water. Thus the milk or milk products mixed with this water processing practices give rise to large amount of waste water. This waste water if not discarded properly will call for environmental crisis in natural water bodies (Kavitha *et al* 2013). Conditions may even be worse due to addition of coloring agents, sweeteners, fruits, proteins etc for fortification or increasing the taste and appearance of processed milk by

products. Being a good source of nutrients the dairy waste water may also allow the growth of pathogens and may lead to objectionable odor and appearance. Thus the need of an hour is to convert this dairy waste water to value added components. In order to establish the process of conversion from '*waste to value'* the present study was deliberated to determine the utility of the buttermilk whey with or without additives for different fermentation period for production of Cephalosporin C from *A. chysogenum.*

The physico- chemical analysis of buttermilk flushing effluent revealed TSS, TDS and TS to be 24.11, 18.73 and 42.84 mg/ ml respectively. The amount of protein estimated by biuret method was found to be 2.6 mg/ml, while that of reducing sugar estimated by DNS was found to be 1mg/ml. Although previous studies report on using high amount of sugars, (Dasari *et al* 2009, Duana *et al* 2012) or addition of rice oil, oleic acid, and linoleic acid which significantly improved the amount of Cephalosporin C production (Kim *et al* 2006). However in the present study 3% lactose was added in the buttermilk flushing effluent as the amount of sugar was found to be very low. The nitrogen content of the effluent determined by kjeldahl method was found to be inadequate; hence the effect of different nitrogen sources like methionine, ammonium sulphate, and cysteine in different combination with 3% lactose was also considered for maximizing the Cephalosporin C production.

Antibiotics being a secondary metabolite are generally produced at the stationary phase. The amount of Cephalosporin C extracted by the end of 4^{th}, 5^{th}, 6^{th}, and 7^{th} day of fermentation revealed that in BM effluent 1.8 fold increase in Cephalosporin C was found from 4^{th} to 7^{th} day of incubation (Fig. 1). Based on the report on Cephalosporin C production at 2.7- 3% sucrose concentration by different workers (Sabbagh *et al*., 2007, Karaffa *et al*. 1997), the present study analyzed the effect of only 3 % lactose on Cephalosporin C production. Studies by Gohar *et al* (2013) show an enhanced production of Cephalosporin C in presence of sucrose, lactose and malt extract. The maximum amount of cephalosporin produced by *A. chrysogenum* was 525.28 mg/l at 2.5% sucrose concentration; however in the present study a maximum of 900µg/ml of Cephalosporin C was obtained at 7^{th} day (Fig. 1) of fermentation in butter milk effluent with 3 % lactose. Although two fractions were collected by column chromatography, the second fraction showed elevated amount of Cephalosporin C (Fig. 1) and was used for further analysis of antibacterial activity.

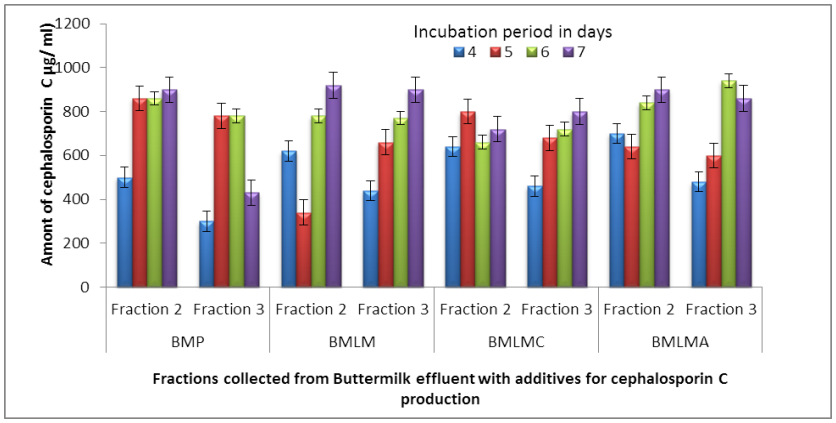

Fig. 1: Production profile of extracted and column purified Cepalosporin C from Buttermilk whey after different period of incubation.

Another study (Raju *et al* 2012) employing *Acremonium chrysogenum* NCIM 893 at 10th day of incubation showed highest yield of Cephalosporin C with fructose in comparison with sorbitol, mannitol, arabinose and glucose. Although in his studies, sucrose or fructose is reported to stimulate the cephalosporin production compared to other carbon sources which are readily metabolized and can suppress the production of cephalosporin. However the present work did not analyzed the effect of different types of sugars and their concentration on Cephalosporin C production utilizing the buttermilk processing waste.

The effect of addition of different organic and in organic nitrogen source on the amount of cephalosporin produced by *A. chrysogenum* is shown in the Fig. 1. It is clear from the results that by the end of 4th day, the highest amount of cephalosporin (700 mg/l) was produced when methionine and ammonium sulphate was added to the culture medium. However by the end of 7th day medium supplemented and methionine and ammonium sulphate also showed enhanced Cephalosporin C production than plain butter milk effluent.

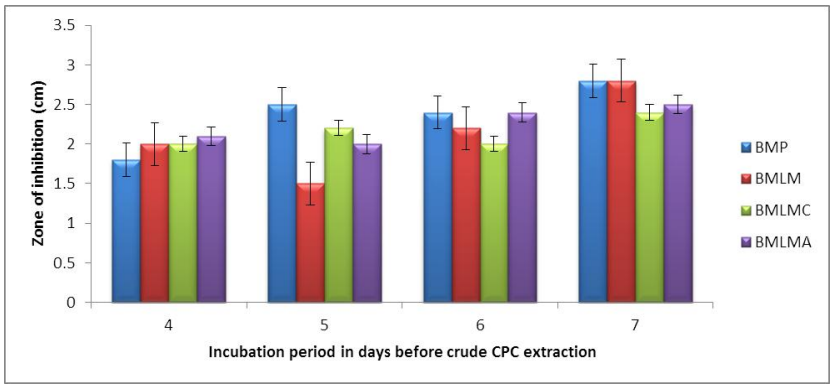

Fig. 2: Zone of inhibition (cm) obtained for extracted and purified fraction 2 from BMP, BMLM, BMLMC and BMLMA medium inoculated with *A. fecalis*.

From 4th to 7th day of fermentation, methionine supplemented medium showed a 1.4 fold increase, while the other two supplemented medium showed an increase of 1.2 fold in Cephalosporin C production. Although methionine, ammonium sulphate is reported as favorable medium constituent for enhancing Cephalosporin C production (Nigam *et al* 2007, Komatsu *et al* 1975, Duana *et al* 2012), this study showed insignificant increase in the Cephalosporin C production in presence of methionine, indicating that other nutritional parameters needs to be refined for maximizing the Cephalosporin C production using the buttermilk whey.

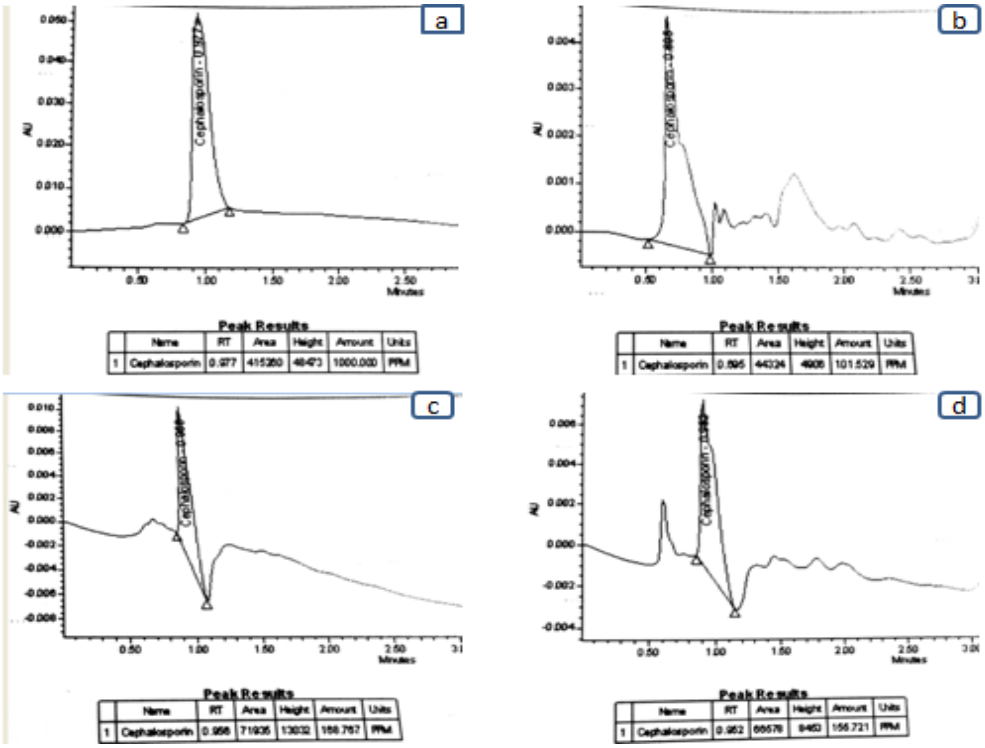

Fig. 3: HPLC chromatogram of extracted fraction 2 from Buttermilk whey medium (a) supplemented with 3% lactose, (b) 3 % lactose and 0.75 % of methionine, (c) 3 % lactose, 0.75 % methionine and 0.1 % cysteine and (d) 3 % lactose, 0.75 % of methionine and 0.4% ammonium sulphate (BMLMA).

The fraction 2 crude antibiotic extracts collected from all the four mediums were purified using the adsorption chromatography. The antibacterial activity was tested against *A. fecalis*. The fractions collected by the end of 4th day from plain and nitrogen supplemented butter milk effluent showed similar zone of inhibition (Fig. 2), however with increase in incubation time (end of 7th day), the amount of Cephalosporin C production increased (Fig 1) and a comparable increase in zone of inhibition was also recorded.

The qualitative analysis of purified antibiotics was carried by HPLC. The standard cephalosporin showed a retention time of 0.999. The chromatogram obtained from fractions collected from BMP, BMLM, BMLMC and BMLMA revealed an enhanced sharp peak and a retention time of 0.977, 0.895, 0.956 and 0.962 resp. confirming the presence Cephalosporin C in the fraction (Fig. 3).

CONCLUSION

As the study is in the preliminary stage further investigation needs to be performed to maximize the Cephalosporin C production utilizing dairy industry waste like buttermilk processing water and with respect to carbon, nitrogen and their concentration, fats, incubation time, pH etc. Thus the study signify the utilization of dairy waste like butter milk whey effluent supplemented with few of the nitrogen sources that may be useful in enhancing the production of Cephalosporin C.

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