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Isolation and Purification of Cephalosporin's from cheese whey

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ABSTRACT

Antibiotics are the secondary metabolites produced by the microbes. The biosynthesis of antibiotics *i.e*. the secondary metabolite is not associated with the growth of mold. In microbial cultures, the production of secondary metabolites and growth related functions do not occur simultaneously. It has been observed that during the production of secondary metabolites, the cell biomass of microbe's increases initially and after certain time, it becomes constant/decreases slightly. It was found out using DNSA method that the lactose content in the above mentioned media was 68.875mg/ml. But then the optimal concentration required for antibiotic production as stated by different published work is 3%. Hence, the concentration of lactose in the media was maintained 3% throughout the experiment by diluting the media 1:2 with distilled water. The nitrogen content found in the media by Kjeldahl distillation method were very meager amounting to only 0.00283% and the protein content being 100 mg/ml. When optimization of parameters for growth was done it was found out that at pH 5.5 maximum growth were seen. This is supported by the fact that in general all the fungal strains thrive well at a lower pH value. The temperature optimum for the growth of *Acremonium chrysogenum* was reported to be 28-30^{o}C on shaker condition at 200 rpm. The pH and temperature further optimized for secondary metabolite production were found to be 7.2 and the temperatures were kept the same. When methionine was added along with lactose, there were a shoot up in the antibiotic production. When the same combination was studied with cysteine being added not much of a difference were seen. This proves that cysteine does not play much of a role in CPC production and does not stimulate CPC biosynthesis to a greater extent.

Keywords: Antibiotics, *Acremonium chrysogenum,* CPC production, PBPs- Pencillin binding protein and cheese whey

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INTRODUCTION

The cephalosporins are a class of β-lactam antibiotics belonging to subgroup-cephams that are originally derived from Acremonium, which were previously known as "Cephalosporium". Cephalosporin compounds were first isolated from cultures of Cephalosporium acremonium from a sewer in Sardinia in 1948 by Italian scientist Giuseppe Brotzu. It were noticed that these cultures produced substances that were effective against Salmonella typhi, the cause of typhoid fever, which had beta-lactamase. The cephalosporin nucleus, 7-aminocephalosporanic acid (7-ACA), were derived from cephalosporin C and proved to be analogous to the penicillin nucleus 6-aminopenicillanic acid, but it were not sufficiently potent for clinical use. Modification of the 7-ACA side-chains resulted in the development of useful antibiotic agents, and the first agent cephalothin (cefalotin) were launched by Eli Lilly in 1964.

Cephalosporin's are bactericidal and have the same mode of action as other beta-lactam antibiotics (such as penicillin's). Cephalosporin disrupts the synthesis of the peptidoglycan layer of bacterial cell walls. The peptidoglycan layer is important for cell wall structural integrity. The final transpeptidation step in the synthesis of the peptidoglycan is facilitated by transpeptidases known as penicillin binding proteins (PBPs). PBPs bind to the D-Ala-D-Ala at the end of muropeptides (peptidoglycan precursors) to crosslink the peptidoglycan. Beta-lactam antibiotics mimic this site and competitively inhibit PBP cross linking of peptidoglycan. First-generation cephalosporins are predominantly active against Gram-positive bacteria, and successive generations have increased activity against Gram-negative bacteria (albeit often with reduced activity against Gram-positive organisms).

Cephalosporins are used to treat infections in many different parts of the body. They are given with other antibiotics as injections to prevent infections before, during, and after surgery. However, cephalosporins do not work for colds, flu, or other virus infections. This product is available in dosage forms such as powder for Solutions, capsules, powder for suspensions, solutions, tablets, injectable tablet forms, suspension tablet and chewable tablets with extended release. The pathway to cephalosporin C is chemically and kinetically well characterized [1]. The common organization of gene cluster for all β-lactam antibiotic producers includes gene pcb AB for ACV synthetase and pcb C for ACV cyclase which are located on chromosome VII. The other genes namely cef D and cef EF present on chromosome I aid in encoding the last step of cephalosporin specific pathway [2]. The initial step in the biosynthesis of cephalosporin involves L-α-aminoadipic acid reacting with L-cysteine to make δ-(L- α-aminoadipyl)-L-cysteine catalyzed by enzyme aminodipyl-cystei-nyl-valine synthetase (i.e. ACV synthetase) in the presence of Mg^{2+} or Mn^{2+} and ATP. The ACVS also catalyses the addition of L-valine to δ-(L- α-aminoadipyl)-L-cysteine to formδ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (i.e.LLD-ACV) [3-5]. The LLD-ACV then undergoes an oxidative ring cyclization by isopenicillin N synthase (IPNS) in the presence of Fe^{2+}, oxygen and ascorbate to form isopenicillin N which in turn is epimerized to penicillin N via the isoepimerase enzyme [3, 6].

Deacetoxycephalosporin C synthase (DAOCS) converts penicillin N to DAOC via an oxidative ring expansion that involves Fe^{2+}, oxygen, ascorbate and α-ketoglutarate [6, 7]. This same enzyme also hydroxylates the DOAC to deacetylcephalosporin C (DAC) and this activity is called deacetoxycephalosporin C hydroxylase (DAOCH) which requires oxygen, α-ketoglutarate and Fe^{2+} to occur [3, 4, 8]. The DAC reacts with acetyl-coenzyme A to form cephalosporin C via the enzyme deacetylcephalosporin C acetyltransferase [9-18].

MATERIAL AND METHODS

(TSS) total suspended solids in effluents:- Collect 40 ml of sample from the stock stored in the flask and divided into two flask each containing 20 ml. Both of the samples were filtered using Whatman's filter paper no.1. The suspended solid matter were scraped and collected from filter paper in a Petri plate already disinfected using 70% alcohol and weighed on a balance. The weight of the solids were then determined. The collected suspended solid were dried in a hot air oven at 110^0C for an hour. After drying, weigh the solids again. Calculate TSS using the formula given.

TDS in effluents: - Collect 40 ml of sample from the stock stored in the flask and divided into two flasks each containing 20 ml. Both of the samples were filtered using what man's filter paper no.1. The suspended solid matter were scraped and collected from filter paper in a petriplate already disinfected using 70% alcohol and weighed on a balance. The weight of the solids were then determined. The collected suspended solid were dried in a hot air oven at 180 ^{o}C for an hour. After drying weigh the solids again. Calculate TDS using the formula given.

Membrane filtration of the media- All the requirements were autoclaved at 121^o C for 15 minutes. The media were then transferred to membrane filter containing a single layer of Whatman's filter paper under sterile conditions. It was then allowed to filter using vacuum pump. The filtered media were then assayed for microbial activity on nutrient agar and Saboroud's agar plates. The results were observed after 48 hours.

Preparation of Standard Curve of reducing sugar by dinitrosalicyclic acid (DNSA) Reagent method - Prepare different concentrations of glucose solution (0.1 mg/ml to 2.0 mg/ml). Take 1 ml of glucose solution and add 3 ml DNSA reagent. Boil this mixture for 20 mins till purple color develops. Dilute the sample and measure OD at 550 nm.

Protein estimation of cheese whey using biuret method- After the preparation of the dilution table start making the stock culture for the standard.
2gm/100 ml , 2000 mg/100ml, 20mg/ml, 200mg/10ml, 0.2gm/10ml
Blank and unknown sample were taken along with standard tubes and analyzed for protein content.

Microbial count analysis of the dairy industry effluent- Prepare the required dilution of sample by sterile dilution technique. Inoculate 0.1 ml of specific concentration in sterile Sabourauds

agar. Spreads the inoculated sample throughout the Petriplate by using glass spreader and let it get solidify. Incubate at 48hrs at room temperature

Total nitrogen estimation in cheese whey by kjedahl method-

Sample preparation: Add 15 g K_2SO_4, 1 ml $CuSO_4.5H_2O$ catalyst solution, and 8–10 boiling chips to digestion flask. Warm milk to 38°C. Weigh warm sample (5-0.1ml) and immediately place in digestion flask. (Note: Weights must be recorded to nearest 0.0001 g.) Add 25 ml H_2SO_4, rinsing any milk on neck of flask into bulb. Flask may be stopper and held for digestion at later time. Distilled water is used as blank.

Determination

(a) Digestion burner setting- Conduct digestion over heating device that can be adjusted to bring 250 ml H_2O at 25°C to rolling boil in 5–6 min. To determine maximum heater setting to be used during digestion, pre heat 30 min (electric) at burner setting to be evaluated. Add 3 or 4 boiling chips to 250 ml H_2O at 25°C and place flask on pre heated burner. Determine heater setting to be used during digestion. That brings water from 25°C to rolling boil in 5–6 min on each burner.

(b) Digestion- Place flask in inclined position with fume ejection system on. Start on setting low enough so that test portion does not foam up neck of Kjeldahl flask. Digest at least 20 min or until white fumes appear in flask. Next, increase burner setting half way to maximum burner set ting determined (a) and heat for 15 min. When digest clears (clear with light blue–green color), continue to boil 1–1.5 hrs at maximum setting (total time 1.8–2.25 hrs). To determine specific boil time needed for analysis conditions in laboratory, select a high protein, high fat milk test sample and determine protein content using different boil times (1–1.5 hrs) after v clearing. A mean protein test increase with increasing (0–1.5 hrs) boil time, becomes constant, and then decreases when boil time is too long. Select boil time that yields maximum protein test. At end of digestion, digest should be clear and free of undigested material. Cool acid digest to room temperature (25 min). Cooled digest should be liquid or liquid with few small crystals. (Large amount of crystallization before addition of water indicates too little residual H_2SO_4 at end of digestion and can result in low test values.) After digest is cooled to room temperature, add 300 ml H_2O to flask and swirl to mix. Let mixture cool to room temperature before distillation. Flasks can be stopper for distillation at later time.

(c) Distillation- Turn on condenser water. Add 50 ml H_3BO_3 solution with indicator to graduated 500ml Erlenmeyer titration flask and place flask under condenser tip so that tip is well below H_3BO_3 solution surface. To room temperature diluted digest, carefully add 75ml 50% NaOH down side wall of Kjeldahl flask with no agitation. NaOH forms clear layer under the diluted digest. Immediately connect flask to distillation bulb on con denser. Vigorously swirl flask to mix con tents thoroughly; heat until all NH_3 has been distilled (150ml distillate; 200ml total volume). Do not leave distillation unattended. Flasks (500ml) may bump at this point. Lower receiving flask and let liquid drain from condenser tip. Turn off distillation heater. H_3BO_3 was titrated

receiving solution with standard 0.1000M HCl solution to first trace of pink. Lighted stir plate may aid visualization of end point.

Fermentation of the Acremonium chrysogenum :- Dilute the cheese whey sample to maintain the lactose contains up to 3%. Add diluted cheese whey sample into the conical flask. Make three flask of 50 ml. Sterilize the all the flask by autoclaving. Then add the following nutrient as following

Nutrient	Amount to be added /50 ml
Lactose	No addition is done in CW. Diluted to 1:2
Ammonium sulphate	0.2 gms
DL- Methioinine	0.375 gms
Cysteine HCL	0.05 gms

Different combination are made as follows along with cheese whey I) Cheese whey plain II) Lactose + DL- Metheoinine III) Lactose + DL- Methioinine + Ammonium sulphate IV) Lactose + DL- Methioinine + Cysteine- HCL . Keep all the fractions at the incubator shaker for 8 days at 30^{o}C at 200 rpm.

Extraction and purification of the cephalosporin c by column chromatography- Collect the 5th, 6th, 7th & 8th day 2 ml fermentation samples. Centrifuge the fermentation samples at 5000 rpm for 10 min to separate the mycelia and fermentation broth. Make the eluent buffer by adding 5 % Butanol in 0.01 N NaOH. Mix 2 grams of activated charcoal powder in 20 ml of water add the glass wool at the bottom of the 2 ml syringe very tightly to avoid leakage of the mixture and put the clamp to maintain the flow rate and holding the water. Add this mixture to the syringe. Add distilled water to from above to settle down the charcoal particles. Keep the column steady and allow it settle down for overnight Next day give washes of distilled water to elute out till 20 ml. Remove water from the column by keeping some amount of water in column to avoid the drying of the column. Pour the broth to the column and allow it pass through the column. Add the eluent buffer to the elute out the antibiotic. Collect five fractions (1 ml). After collection of fraction they have to be kept in ice to avoid the degeneration of the antibiotic. Keep this fraction open in laminar flow to evaporation of the butanol. After complete evaporation of the butanol add ethanol upto 50%. Then again complete evaporate the ethanol and do the antibiotic assay of the fractions.

Antibiotic Assay:-Prepare sterile nutrient agar plate. Spread the test organisms of O.D. 0.01 using the sterile spreader. Bore the agar using sterile 10 mm borer. Add 100 µl of sample into the wells. Keep the plates overnight for incubation at RT. Observe the result for the zone of inhibition. Perform the HPLC for the qualitative and quantitative analysis.

High Performance Liquid Chromatography- Sample preparation- The sample was dissolved in 0.5% acetic acid in acetonitrile to get the concentration of 1mg/ml in respect to standard. Further dilutions were made with MP. Standard preparation- 1mg/ml solution of each antibiotic prepared in 0.5 % acetic acid in acetonitrile pH 3.6. Add 20 µg of the standard to the injection

system of the HPLC system maintain the flow rate (isocratic) 2ml/min by maintaining the pressure detection of the standard done at UV-254nm. Analyze the graph by using the HPLC chromatogram analyzing system. The same procedure was repeated for the samples and comparsion was done with the chromatograms

RESULTS AND DISCUSSION

Antibiotics are the secondary metabolites produced by the microbes. The biosynthesis of antibiotics i.e. the secondary metabolite is not associated with the growth of mold. In microbial cultures, the production of secondary metabolites and growth related functions do not occur simultaneously. It has been observed that during the production of secondary metabolites, the cell biomass of microbe's increases initially and after certain time, it becomes constant/decreases slightly. Production varies with the constituents of the media and the stage of the culture development. The secondary metabolites accumulate only after the growth phase (tropophase) i.e. when the culture attains a specific growth rate. Secondary metabolites are often called idiolite since they are produced in the idiophase. This investigation on the biosynthesis of CPC was attempted with an aim to find out its production as it is a very important beta lactam antibiotic with a broad-spectrum activity. A thorough literature review was performed and it was noticed that though a number of highly productive strains and a number of different strategies have been used for better yield, not much attention has been given to produce this antibiotic using biological wastes as media. In addition, a number of other reports are available on the production of CPC using complex medium.

The basis of the work on cheese whey started with the estimation of basic nutrients and elements. The estimation of lactose, nitrogen and protein content was done. It was found out using DNSA method that the lactose content in the above mentioned media was 68.875mg/ml. But then the optimal concentration required for antibiotic production as stated by different published work is 3%. Hence, the concentration of lactose in the media was maintained 3% throughout the whole experiment by diluting the media 1:2 with distilled water. The nitrogen content found in the media by Kjeldahl distillation method was very meager amounting to only 0.00283% and the protein content being 100 mg/ml. When optimization of parameters for growth was done it was found out that at pH 5.5 maximum growths was seen. This is supported by the fact that in general all the fungal strains thrive well at a lower pH value. The temperature optimum for the growth of Acremonium chrysogenum was reported to be 28-30^{o}C on shaker condition at 200 rpm. As has been mentioned earlier, growth and secondary metabolite production does not occur simultaneously. So the optimized parameters for growth may or may not be suitable for cephalosporin C production [Table1-5].

Table 1:- Dry weight of the sample (Cheese whey flushing effluent)

Sample types	**Cheese whey flushing effluent**	
Observations	Sample 1	Sample 2
Weight of Petri plate	45.0392	46.8942
Weight of sample taken	0.76440	0.5444
Weight after drying	45.6100	47.1550

Calculations: - Formula: - (W1-W2) ×1000 mg/ml

Table-2:- Total Suspended Solids

Sample types	**Cheese Whey effluent**	
Results	Sample 1	Sample 2
TSS (mg/ml)	19.30	28.93

Table 3 :- Dry Weight (Butter Milk flushing effluent)

Sample types	**Butter Milk flushing effluent**	
Observations	Sample 1	Sample 2
Weight of Petri plate	44.5322	44.0333
Weight of sample taken	1.0005	1.0030
Weight after drying	44.5227	45.0345

Formula: - (W1-W2) ×1000 mg/ml
Sample volume, Where W1= Weight of sample before drying (Here Weight of Petri Plate+ Weight of Sample added to Petri plate. W2= weight of sample after drying

Table 4 :- TDS effluent (Butter Milk flushing effluent)

Sample types	**Butter Milk flushing effluent**	
Results	Sample 1	Sample 2
TDS (mg/ml)	18.90	18.56

TS= TSS+TDS

Table- 5 Total Suspended (Butter Milk flushing effluent)

Sample types	**Butter Milk flushing effluent**	
Results	Sample 1	Sample 2
TS (mg/ml)	39.69	69.265

The pH and temperature further optimized for secondary metabolite production was found to be 7.2 and the temperature was kept the same. At this temperature and PH antibiotic production was seen to be maximum. In order to see the effect on biosynthesis of antibiotic, various components were added to cheese whey and their effects were compared to plain minimal media containing no additions. The effect of four different components lactose, DL-methionine, cysteine and ammonium sulphate was studied. As has been mentioned earlier, lactose acts as a carbohydrate source and DL-methionine and cysteine are precursors for Cephalosporin biosynthesis. Ammonium sulphate, on the other hand, acts as a source of nitrogen required for mycelia formation. After 96 hours of growth, the media was assayed for antibiotic and it was seen that when compared to plain cheese whey, there was much of an increase in antibiotic production on addition of Methionine. On the other hand, media

containing the combination of lactose and DL-Methionine and ammonium sulphate showed quite less production after 96 hours compared to only lactose and Dl- methioine. This might have happened due to the fact that cheese whey had very high amount of nitrogen initially but then less addition of ammonium sulphate is required as source of nitrogen for CPC production. Similar kinds of results were seen after 120, 144 and 168 hours of incubation with the latter combination. Although after 120 hours, lactose, methionine and cysteine combination also showed an increase in the production of antibiotic [Fig 1-13].

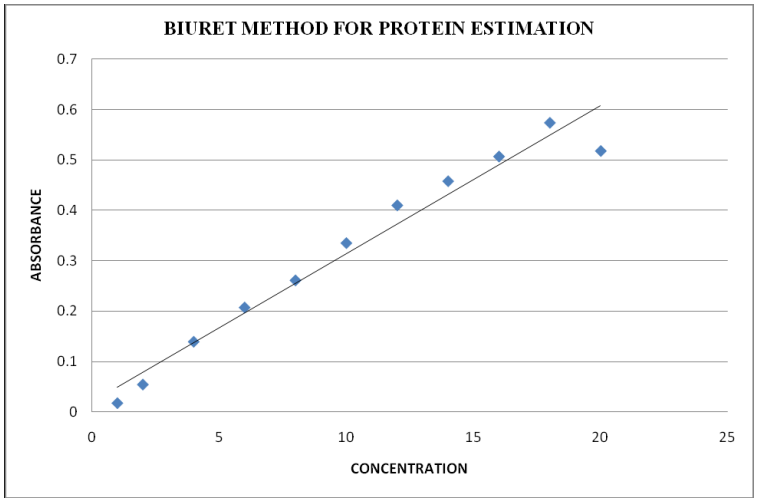

Fig 1: Concentration of the Protein in cheese whey form graph is found to be 100 mg /ml

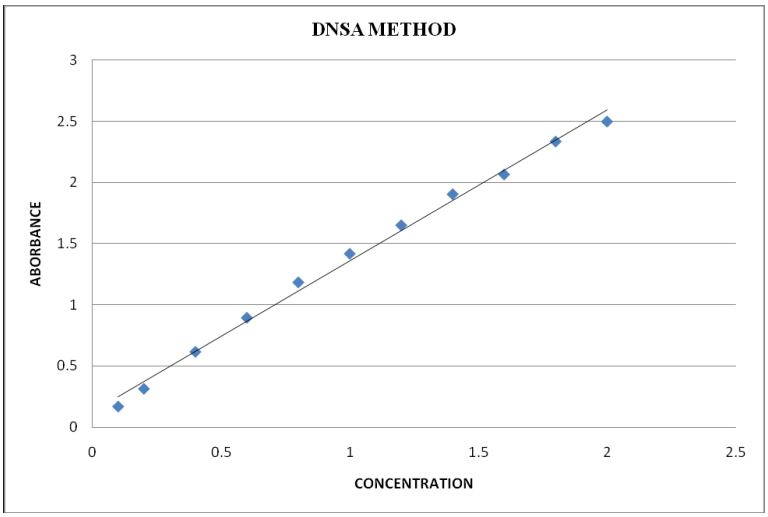

Fig 2: O.D if the cheese whey sample is diluted to the (1:50) is 1.73, therefore concentration of the lactose from the graph is found to be 1.45. There for by multiplying by dilution factor concentration of lactose from the sample was found to be 68.875mg/ml.

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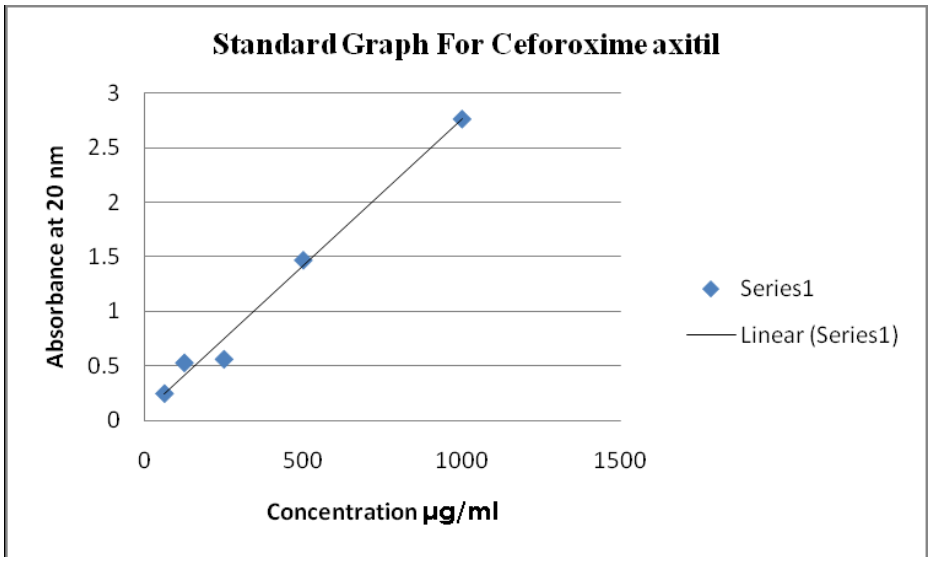

Fig 3 : - Standard Cefuroxime Axiti

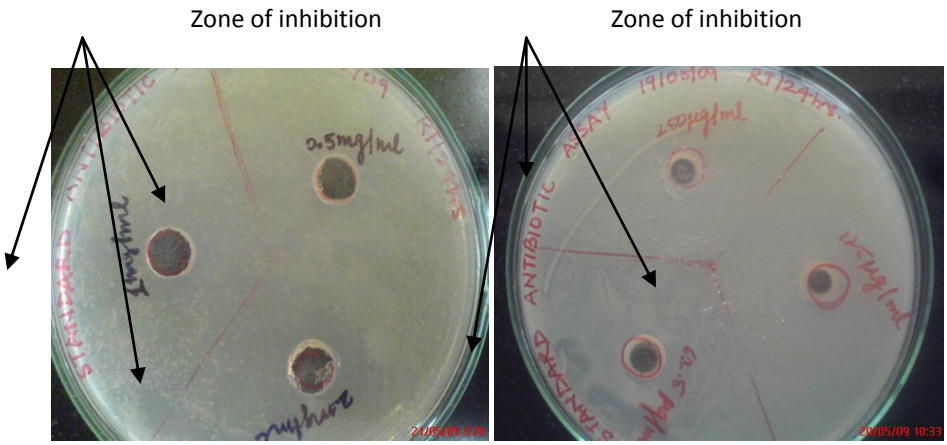

Fig 4 : - Zone of inhibition

The Fermentation Sample after Purification

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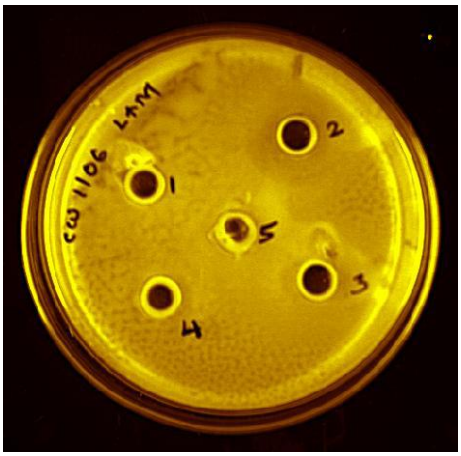

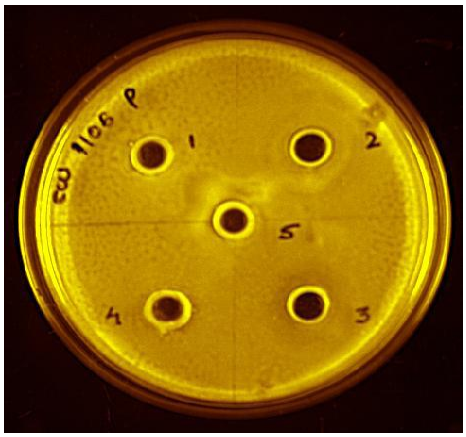

Fig 5 :- Cheese whey (L+M), 120 Hrs. **Fig 6 :- Cheese whey (P), 120 Hrs**

Zone of inhibition

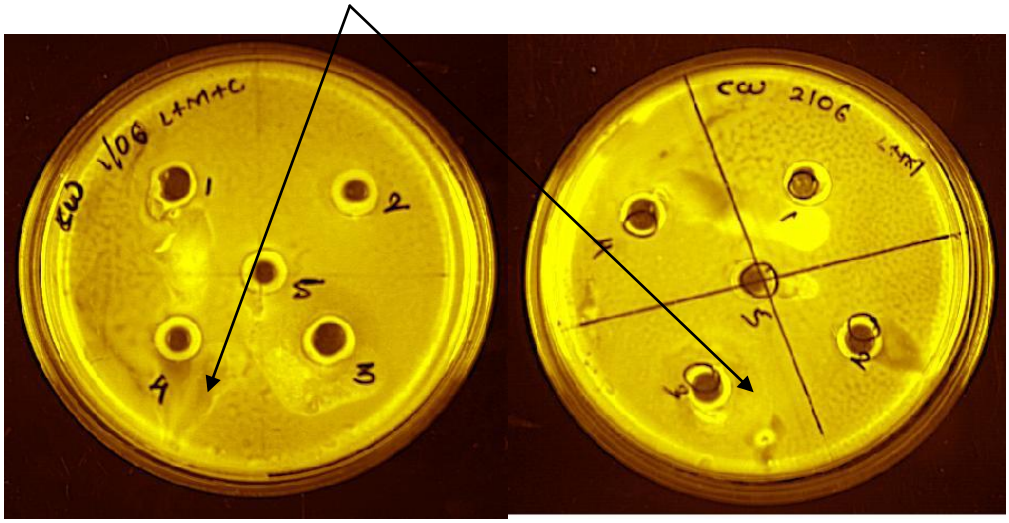

Fig 7 :- Cheese whey (L+M+C), 120 hrs **Fig 8 :- Cheese whey (L+M), 144 Hrs**

Zone of inhibition

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Fig 9 :- Sample – Cheese whey (L+M), 168 Hrs

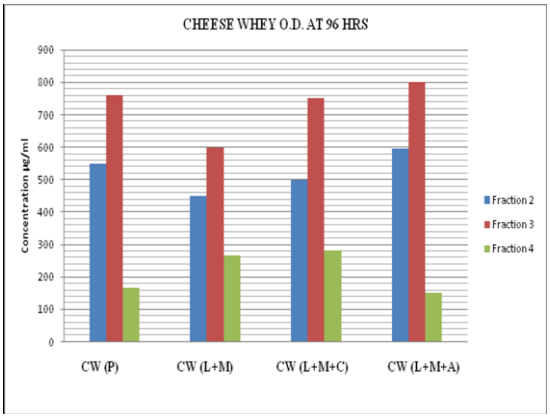

Fig 10 :- Cheese Whey OD at 96hrs.

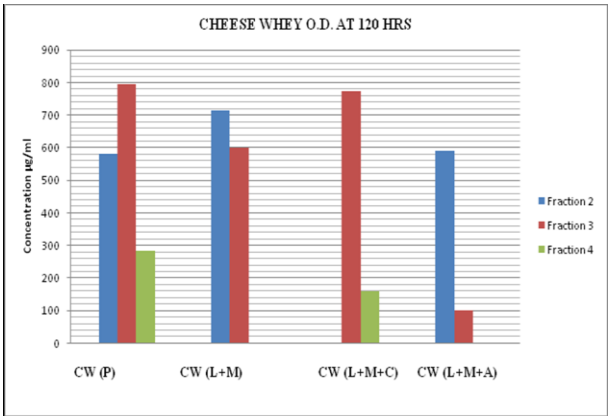

Fig 11 :- Cheese Whey OD at 120hrs.

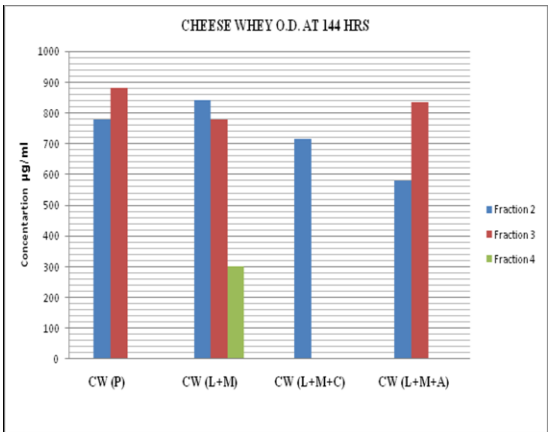

Fig 12 :- Cheese Whey OD at 144hrs

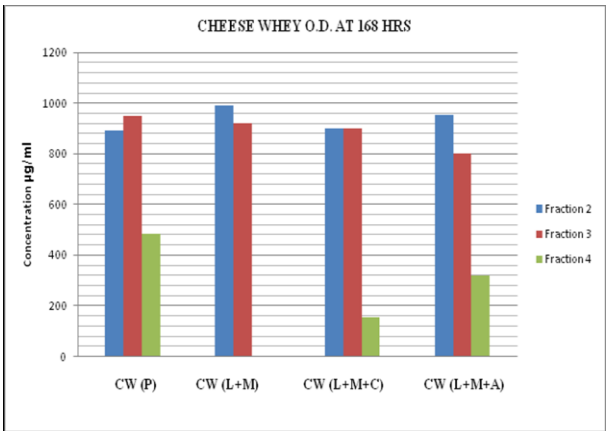

Fig 13 :- Cheese Whey OD at 168hrs

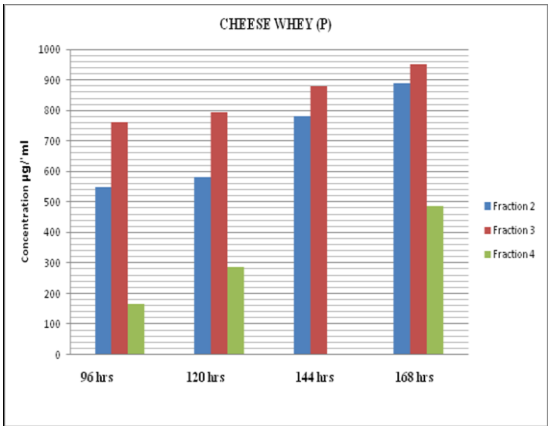

Fig 14 :- Cheese Whey (P)

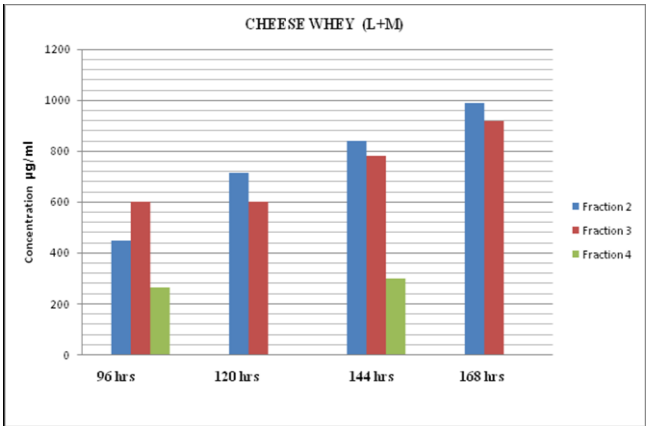

Fig 15 :- Cheese Whey (L+M)

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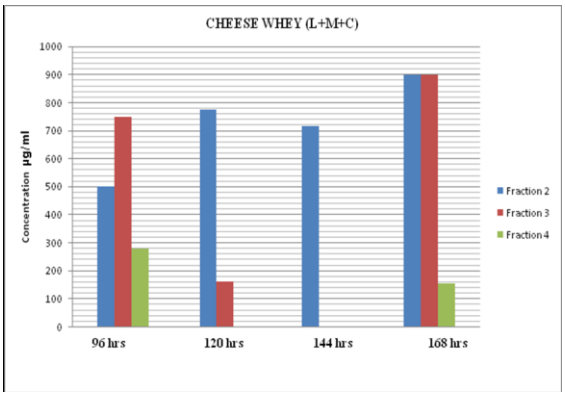


Fig 16:- Cheese Whey (L+M+ C)

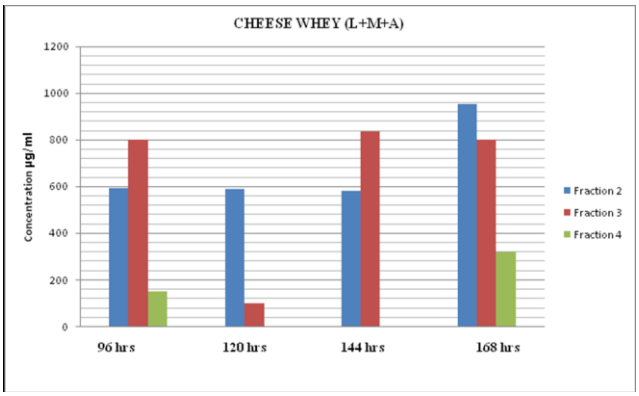
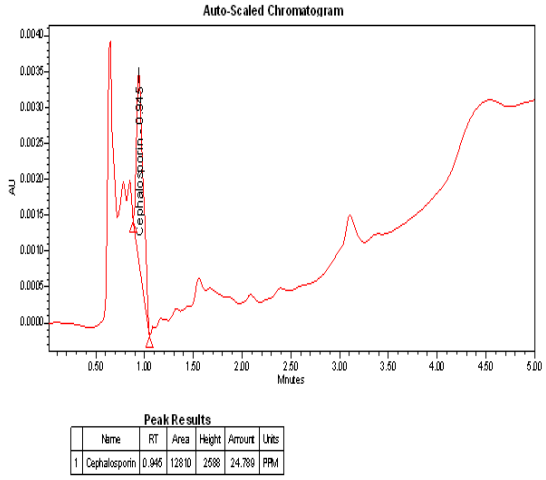
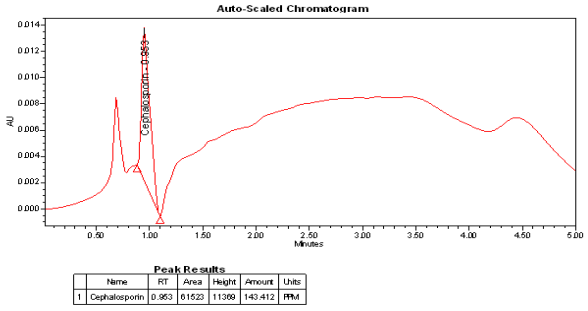


Fig 17 :- Cheese Whey (L + M+ A)



Peak Results

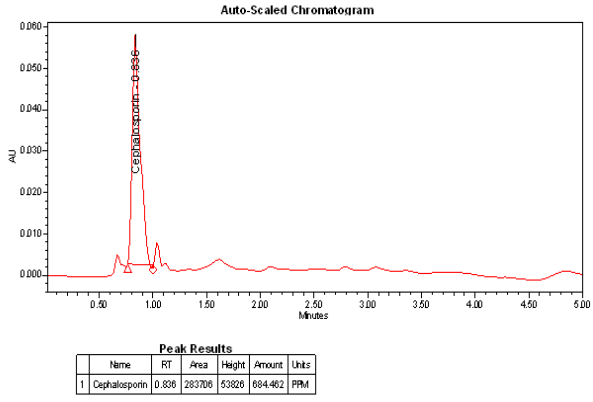
	Name	RT	Area	Height	Amount	Units
1	Cephalosporin	0.946	12810	2688	24.789	PPM



Peak Results

	Name	RT	Area	Height	Amount	Units
1	Cephalosporin	0.953	61523	11369	143.412	PPM

Fig 18 :- CW (L+M+C), 120 hrs, Fraction- 3

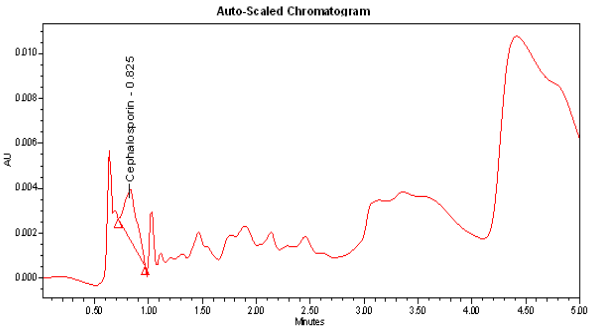


Peak Results

	Name	RT	Area	Height	Amount	Units
1	Cephalosporin	0.836	283706	53826	684.462	PPM

Fig 19:- CW (L+M), 120 hrs, Fraction -2

Fig 20 :- CW (L+M), 168 hrs, Fraction 3



Peak Results

	Name	RT	Area	Height	Amount	Units
1	Cephalosporin	0.825	17631	2208	36.527	PPM

Fig 21 :- CW (L+M), 144 hrs, Fraction-3

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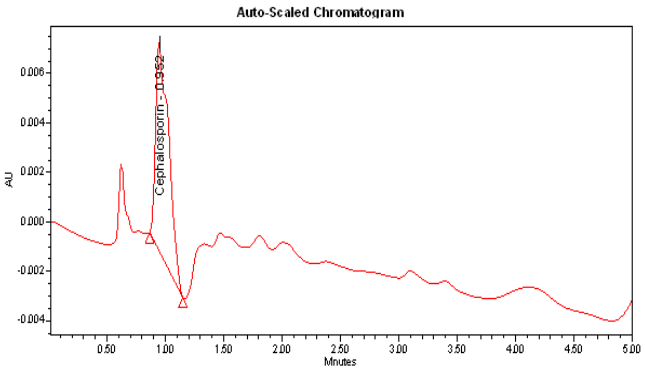
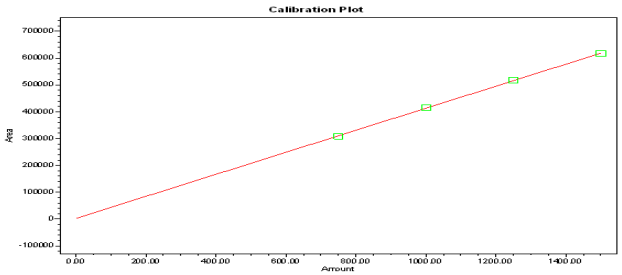


Peak Results

	Name	RT	Area	Height	Amount	Units
1	Cephalosporin	0.952	66578	8463	155.721	PPM



Peak: Cephalosporin

	Name	Level	X Value	Response	Calc. Value	% Deviation	Manual	Ignore
1	Cephalosporin		750.000000	308966.379222	746.974083	-0.537	No	No
2	Cephalosporin		1000.000000	415260.475856	1004.816258	0.482	No	No
3	Cephalosporin		1250.000000	516949.846846	1252.445236	0.196	No	No
4	Cephalosporin		1500.000000	617280.048945	1496.764423	-0.216	No	No

Fig 22 :- CW (P), 120 hrs, Fraction-3
Fig 23 :- Calibration Plot

This gradual increase in the antibiotic was seen due to the stimulatory effect of methionine which acts as a precursor and adds sulphur to the antibiotic. When individual production was analyzed, it was found that when compared to plain cheese whey, the combination of lactose and DL- methionine gave better yield. When methionine was added along with lactose, there was a shoot up in the antibiotic production. When the same combination was studied with cysteine being added not much of a difference was seen. This proves that cysteine does not play much of a role in CPC production and does not stimulate CPC biosynthesis to a greater extent. After CPC was produced, its assay was carried. The media containing the antibiotic was assayed and instead of a zone of inhibition, a zone of stimulatory growth was observed. This can be related to the fact that cheese whey already has nutrients and also the antibiotic in the media was in diluted form which seemed to have masked its effect, thereby giving a zone of stimulation with the test culture Alcaligenes faecalis O.D. 0.1 [Fig 14-17].

In order to rule out this possibility, the antibiotic was purified using adsorption chromatography with activated charcoal and again antibiotic assay was carried out. This assay gave proper zones of inhibition. Further in order to verify the presence of antibiotic in these fractions, spectrophotometric readings at 260 nm was taken along with standard Cefuroxime axetil. The readings were found to be in accordance with the standard. Futher qualitative and quantitative analysis of the antibiotic was carried out using HPLC. The chromatogram obtained with lactose, methionine combination gave an enhanced sharp peak comparable with the standard confirming the presence of CPC production. Similar peaks were obtained with samples of combinations of lactose and methionine [Fig 18-23].

The above studies conclude that for better yield of CPC, biological wastes such as cheese whey and others could be utilized to fulfill the nutritional requirement of mould at their optimal conditions. It was found that methionine at the concentration of 0.4% gave higher yields of antibiotic along with cheese whey medium. Cysteine- HCl is nor playing much important role in production of antibiotic whereas nitrogen content in media play very important role in antibiotic formation. High nitrogen content in nutrient medium may lead to the feedback inhibition on antibiotic production.

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