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Process development and optimization for production of 3-O-Glycosyl colchicinoid

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ABSTRACT

The study was conducted for exploring the biotransformation of colchicinoid compounds into corresponding 3-O-Glycosyl derivative by means of *Bacillus megaterium* strain. The experiments were conducted to show the effect of colchicines on different bacterial species and biotransformation of colchicines into 3-O-glycosyl derivative by means of selected microbial strain of *B. megaterium*. The process provides colchicinoid compound glycosylated at C-3 of aromatic ring. It involves media optimization and process optimization approach for obtaining high yield. Preliminary studied was the effect of colchicine on other bacterial species *E. coli* & *B. subtilis*. Their growth was inhibited due to high concentrations of colchicines as observed by zoographic microscopy after interval of 48 hrs. The effect was observed using two culturing conditions first in liquid culture observed through photo. Then using *B. megaterium* considering 25ml of sterile-broth with different ranges of colchicines 12.5 mg, 17.5mg, 22.5mg and 2 ml of culture goes for biotransformation observed through TLC using 10% alcoholic sulphuric acid. The effect of different culture conditions was studied on the production of 3-O-glycocolchicine.

KEYWORDS

Colchicinoids;
Bacillus megatarium;
Biotransformation;
3-O-Glycosylation.

INTRODUCTION

"3-O-glycosylcolchicinoid" is the precursor for a muscle pain relief drug. Patent describes a process for production of 3-O-glycosyl derivative by biotransformation of 'colchicinoids'. Colchicinoids are the colchicines derivatives and are found in abundance in nature likewise extracted from a native Jordanian meadow saffron[1].

The range of media requirements and conditions is already described in the patent. We have optimized the protocol and thus have developed the fermentation process for carrying out biotransformation on large scale[2].

Bacillus megaterium is a rod-shaped, Gram-positive, endosperm forming, species of bacteria used as a soil inoculant in agriculture and horticulture. Bacterium is arranged into the streptobacillus form[3].

Bacillus megaterium is a rod shaped bacteria and

one of the largest eubacteria found in soil. Groups of the bacteria are often found in chains where the cells are joined together by polysaccharides on the cell walls. *Bacillus megaterium* is able to survive in some extreme conditions such as desert environments due to the spores it forms[4].

Colchicinoid compounds are transformed into the corresponding 3-O-glycosyl derivatives by means of bacillus megaterium strains. Such a transformation takes place in a very short time, and is characterized by surprisingly high yield[1,5].

The effects of colchicine and its analogs on the carrageenan-induced footpad edema in rats were investigated. The anti-inflammatory effects of colchicine analogs were measured at 3 and 5 hr after the carrageenan injection. Colchicine, 1-demethylcolchicine and 3-demethylcolchicine markedly inhibited the carrageenan edema whereas 2-demethylcolchicine was much less active. Thiocolchicinoids, having a thiomethyl group at C-10 instead of a methoxy group, were considerably less potent. These results suggest that the presence of methoxy groups at C-2 and C-10 in colchicine is necessary to maintain anti-inflammatory activity. Inactivity of deacetylcolchicine indicates that substitution of the amino group at C-7 with electron withdrawing groups is also important. Significant inhibition of carrageenan edema and strong binding to tubulin in vitro were manifested by colchicine, 3-demethylcolchicine, N-butyryldeacetylcolchicine and colchifoline. On the other hand, N-carbethoxydeacetylcolchicine which did bind well to tubulin did not show much effect on the carrageenan edema. These results suggest that the anti-inflammatory action of colchicinoids may not be regulated through the microtubule system[2,6]. The authors have investigated the studies on Jordanian *Colchicum* species; the biologically active components of *Colchicum brachyphyllum* were pursued. Using bioactivity-directed fractionation, nine colchicinoids were isolated and characterized. One of these has a novel ring system, to which the researchers have ascribed the trivial name demecolcinone, and represents the first naturally occurring dextrorotatory colchicinoid. Another isolated compound was a new colchicinoid analogue, 2, 3-didemethyldemecolcine, while the remaining seven known colchicinoids were new to the species: colchicines, 3-demethylcolchicine, cornigerine, γ-lumicolchicine, androbiphenyline, demecolcine, and 3-demethyldemecolcine. The brine shrimp lethality test was used to direct the isolation of these colchicinoids. Moreover, all pure compounds were evaluated for cytotoxicity against a human cancer cell panel, for antimicrobial activity in an array of bacteria and fungi (including yeast), and for their potential to be allosteric modulators of the γ-aminobutyric acid type A receptor[3]. *Bacillus megaterium* has been industrially employed for more than 50 years, as it possesses some very useful and unusual enzymes and a high capacity for the production of exoenzymes. It is also a desirable cloning host for the production of intact proteins, as it does not possess external alkaline proteases and can stably maintain a variety of plasmid vectors. Genetic tools for this species include transducing phages and several hundred mutants covering the processes of biosynthesis, catabolism, division, sporulation, germination, antibiotic resistance, and recombination. The seven plasmids of *B. megaterium* strain QM B1551 contain several unusual metabolic genes that may be useful in bioremediation. *B. megaterium* in industry, sporulation and protease-deficient as well as UV-sensitive mutants were constructed for a broader application. The genome sequence of two different strains, plasmid less DSM319 and QM B1551 carrying seven natural plasmids, is now available. These sequences allow for a systems biotechnology optimization of the production host *B. megaterium*. Altogether, a "toolbox" of hundreds of genetically characterized strains, genetic methods, vectors, hosts, and genomic sequences make *B. megaterium* an ideal Organism for industries[4].

Colchicine, (*S*)-*N*-(5, 6, 7, 9-tetrahydro-1, 2, 3, 10-tetramethoxy-9-oxobenzo-(a (-heptalen-7-yl)-acetamide, is the main alkaloid contained in *Colchicum autumnale* (meadow saffron). There are known colorimetric, spectrophotometric, volumetric, potentiometric, voltametric, gravimetric and various chromatographic methods for quantitative determination of colchicine, each of them presenting a series of advantages and disadvantages. As an alternative, we proposed the use of a densitometric determination for colchicine allowing the determination of this alkaloid from pharmaceutical products, as well from seeds of meadow saffron. The total alkaloid extract was separated by Thin-Layer Chromatography using Silicagel 60F254 layers

and a mixture of chloroform: acetone: diethyl amine (5:4:1) as mobile phase. The same conditions were used for the determination from pharmaceutical products. Densitometric measurements were carried out at the absorption maximum 350 nm of colchicine, the determinations being made by reflectance and by fluorescence. The peaks were optimized regarding to their area and shape by varying four scanning parameters (slit width and height, number of measurements and scanning speed). We established the calibration plot using pure colchicine in the range 50–600 ngmL^{-1}. The method was characterized by validation parameters (linearity, accuracy, fidelity, sensitivity) and it was established in accordance with an HPLC method and an official quantitative determination from the Romanian Pharmacopoeia X edition respectively[5-8].

SIGNIFICANCE OF THE INVENTION

Colchicine is a toxic natural product and secondary metabolite, originally extracted from plants of the genus *Colchicum* (autumn crocus, *Colchicum autumnale*, also known as "meadow saffron"). This research is highly significant cause of diverse effects of colchicine as described:[9-13]

Tubulin binding and anti inflammatory activity of colchicines makes it more useful.

Colchicine can arrest cell division and used originally to treat rheumatic complaints, especially gout, still finds use for these purposes today despite dosing issues concerning its toxicity. It is also prescribed for its cathartic and emetic effects.

Colchicines' present medicinal use is in the treatment of gout and familial Mediterranean fever; it is used as initial treatment for pericarditis and preventing recurrences of the condition. It is also being investigated for its use as an anti-cancer drug.

MATERIALS AND METHODS

Colchinocides purchased from the Bio India Biological (BIB) Corporation and bacterial strain *Bacillus megatarium* from Micronial Type Culture Collection Gene Bank (MTCC), Chandigarh India. Nutrition broth and nutrition agar was purchased from the leading biochemical manufactures in India. Media preparation done employing LB agar sterilization at 120 °C.20' at pH 7 with triptone 10g/l, yeast extracts 5g/l, NaCl 10g/l and agar 15g/l[14-18].

Broth ST Sterilization was done at 121°C. 20' and pH 7 with Glucose 20g/l, Glycerol 10g/l, Peptone 15g/l, Yeast extract 5g/l, NaCl 3g/l, NH„ Cl 3g/l, K, HPO„ 8g/l, KH, PO„ 3g/l and MgSO„ .7H, O 0.5g/l. Inoculation of strain was carried out from the ampoule to prepare culture[19,20].

EXPERIMENTAL

Colchicine concentration was studied on various strains viz. *E. coli* and *Bacillus subtillis* employing test tubes and beakers. Submerged culturing was performed. Bacterial strains and colchicine was put in test tubes. It was allowed to mix and react. Spectrophotometer was employed for analyzing the response. The difference in optical density confirmed the reaction involved[21].

Media culturing incubating bacterial strains was performed for checking bacterial sustainability response to colchicines concentration. For this study, flasks, beakers. Petri plates, incubator and laminar flow were employed. Colchicine, *E. coli, Bacillus subtillis,* utilized for these experiments. Media was prepared using all the mentioned constituents. Streaking was done on media in different 14 plates. Then the colchicine was added onto the plates. The effect on the reaction was observed[22].

α-amylase activity in biotransformation of colchicines was evaluated using the flasks, beakers, pipettes, shaker incubator. In this study 2 mg of α-amylase was dissolved in 5 mg of colchicine, was introduced into the shaker incubator, and was allowed to dissolve for an hour. The readings were taken during this dissolution. Analysis was performed employing the thin layer chromatography and the spots were identified in iodine fuming chamber by referring standards[23].

For studying the effect of *Bacillus megaterium* on colchicine biotransformation, nutrient broth, bacillus strain, culture media and colchicine were utilized. Liquid and media culturing of strain was performed in the test tubes. One with colchicine whiles other with bacillus+ colchicine. Detection was observed with spectrophotometer by measuring absorbance[24].

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Plates were prepared using silica gel and the spotting was done using lancet. The plates were placed in the TLC chamber containing acetone: ethyl acetate: water (5:4:1). The sample spots travelled corresponding to respective Rf values. The spots were visualized in iodine fuming chamber.

The effect of *B. megaterium* on different colchicines compositions was studied employing colchicine, bacterial culture, ST-broth, flask, pipettes considering seven solutions 1-7[25-28].

The action of *B. megaterium* on colchicine biotransformation was experimented employing the ST-broth, bacterial culture, colchicine, 100% ethanol, laminar air flow, spectrophotometer and microbial centrifuge[6,7].

Stock solution was prepared by adding 25ml broth to 25ml ethanol and 1mg colchicine corresponding 0.02mg/ml containing different dilution of 100% ethanol with the dilution factor = (0.02/x-1) with different range of concentration ranging 0.001,0.002,0.003, 0.004,0.005,0.006,0.007,0.008,0.009,0.010,0.011, 0.012 mg/ml. Thus by providing different set of concentration a standard curve is been generated with concentration and absorbance at wavelength of 350 nm[25-28].

The quantification of different colchicines concentration solutions corresponding 0.5, 0.7, 0.9 mg/ml with ST-broth and *B. megatreium* was done. Accordingly absorbance was calculated in different time period by dissolving each concentration with respective dilution factor. The procedure includes centrifugation 1.5ml of culture and then 1ml of supernatant is been used for dilution with respective dilution factors[29].

Three set up were constructed: For making dilution the 100% ethanol was added to different solutions in respective ranges so that it lies in between standard curve. 0.5mg/ml - 49ml of 100% ethanol: 0.7mg/ml – 69ml of 100% ethanol; 0.9mg/ml- 89ml of 100% ethanol respectively.

RESULTS

The effect of colchicines concentration on bacterial strains was observed through microscope using gram staining techniques. The results obtained have shown that colchicne retarded the growth of strains *bacillus*. Colchicines above certain concentration have proven to be fatal for bacterial species growth.

LB-agar mediated culturing of *E.coli* and *Bacillus subtillis* has shown retarded growth when colchicine was higher than 0.5mg/ml. As a result the bacterial colonies were found to be killed after 48 h[6,8,9].

These studies have shown that colchicine was having negative growth effect on different bacterial species. It has shown no effect of direct usage of α-amylase in the biotransformation process on colchicine. The presented standard curves have shown linear change of absorbance with respective change in concentration of the solution[30].

DISCUSSION

Specifically, the microorganisms usable in the present invention was selected starting from collection cultures obtained from strain deposit centers, or from soil samples of various origin, or from preselected industrial strains, by selective recovery on different agar media containing an organic nitrogen source (peptones, yeast extracts, meat extracts, asparagine, etc), a carbon source (glycerin, starch, maltose, glucose, etc.), with pH 5 to 8, preferably 6- 7. The incubation temperature ranges from 20° to 45*C, preferably 28°-40°C[6,8].

The ability of the culture of growing in the presence of toxic concentrations of the colchiconic substrate to be transformed was evaluated by techniques of scalar dilution and plating in parallel, on different agarized substrates. The colonies capable of growing in the described conditions were withdrawn aseptically in sterile atmosphere and placed on different agarized media, to verify their purity and the homogeneity of growth[6,8].

The culture media used for the conservation of the culture were typical microbiological substrates, containing organic nitrogen sources (peptones, yeast extracts, tryptone, meat extracts, etc.), a carbon source (glucose, maltose, glycerin, etc.), at pH 5 to 8, preferably 6-7. The incubation temperature ranges from 20° to 45°C, preferably 28°-40°C[6-9].

The selected microorganisms were then assayed for the capability of growing in submerged culture, in the presence of colchiconic compounds, and of transforming the latter into the corresponding 3-glycosyl derivatives[6-9].

Said assays were carried out in 100 ml flasks containing 20 ml of liquid medium, with different medium formulations, comprising one or more organic nitrogen sources (yeast extracts, peptones, tryptone, casein hydrolysates, etc.), one or more carbon sources (glucose, glycerol, starch, etc.), inorganic phosphorous and nitrogen sources, and inorganic salts of various ions (K+, Na+, Mg++, Ca++, Fe++, Mn++, etc.)[3,30].

The culture samples could optionally be subjected to mutagenic treatments, by means of the conventional mutagenesis techniques (irradiation with UV rays, etc.) to induce mutants having a specific bioconversion activity. The selected bacterial microorganisms were capable of affecting the biotransformation. It was grown on both solid and liquid culture substrates, containing one or more organic nitrogen sources, preferably yeast extract, peptone, tryptone, casein hydrolysates, cornsteep liquor, etc. Carbon sources useful for the growth and the biotransformation were glucose, fructose, saccharose, glycerol, malt extract, etc., preferably glucose, fructose and glycerin. The culture medium contains moreover inorganic phosphorous sources and salts of K+, Na+, Mg++, NH4+, etc[3,6-10].

The biotransformation of the invention was based on an enzyme conversion, which starts during the growth exponential phase and continues with a parallel progression to that of the growth; the maximum levels of conversion to 3-O-glycosyl derivative (up to 95%) were reached within the first 48-72 hours, depending on the addition time of the substrate. The regioselectivity of the biotransformation was absolute. No presence of 2-O-glycosyl derivatives was evidenced in the culture samples. The resulting products are exclusively extracellular[3].

The substrate to be transformed could be added in acetone or alcohol solution, in alcohol-water mixtures, in dioxane, etc. The biotransformation of the invention could be scaled up to fermenter level, keeping the culture conditions unchanged, in particular as far as culture medium, temperature and processing times are concerned[3].

In order to obtain good growths, adequate levels of stirring-aeration are important, in particular aeration levels of 1-2 liters of air per liter of culture per minute (vvm), preferably of 1, 5-2 vvm are required[3].

The products resulting from the bioconversion were extracted from the culture broths after separation of the biomass from the liquid fraction by centrifugation. The supernatant, or microfiltration and permeate was recovered. The culture was treated with alcohols, in view of an optimum recovery of the product[3].

The purification and the recovery of the biotransformation products were carried out using chromatographic techniques for the separation on absorption resins and elution with methanol. The aqueous methanol solutions containing the product were further purified by extraction with lipophilic organic solvents, preferably with methylene chloride. After further treatments with mixtures of alcohols and organic solvents, the product was obtained in the pure state from the resulting alcohol solutions by crystallization. Glucose can be replaced by other sugars, such as fructose or galactose, without causing the loss of the glycosyl transferase activity[3].

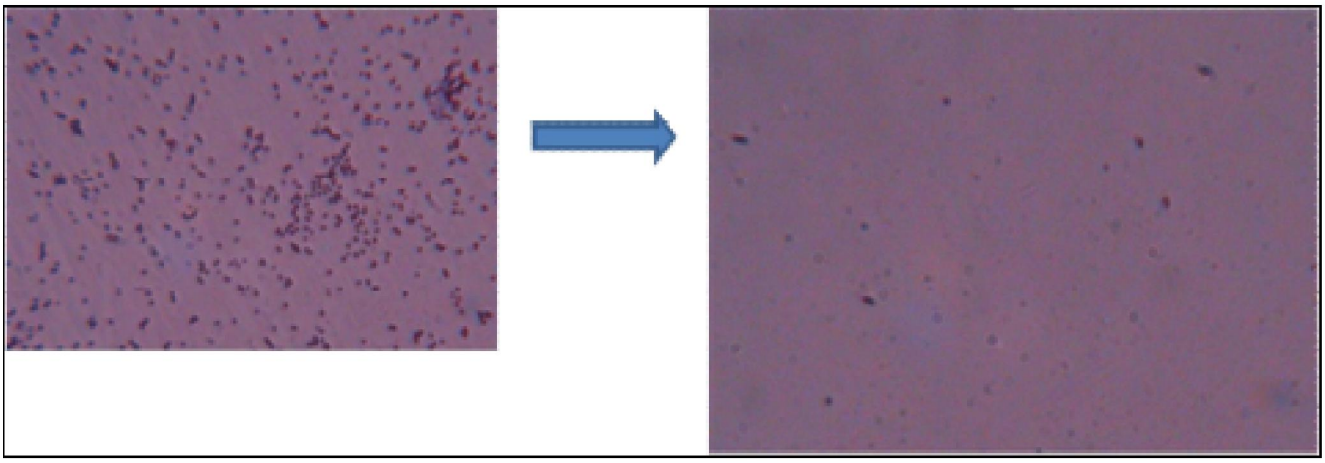

Figure 1 : Bacterial culture and bacterial culture with colchicine

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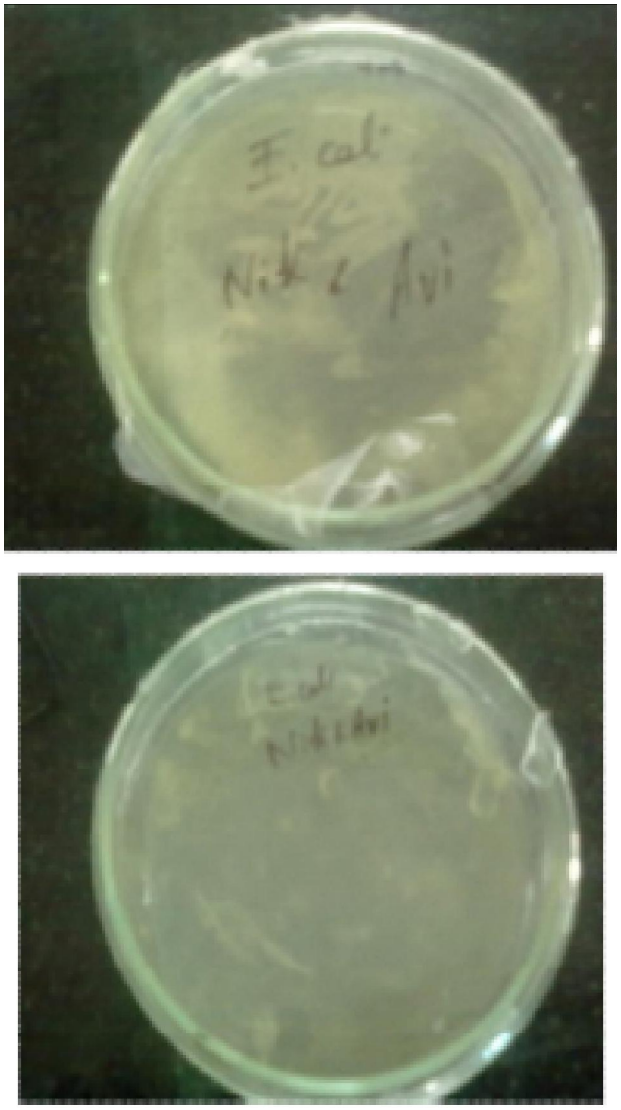

Figure 2 : *E. coli* culture and *E. coli* culture with colchicines

Figure 3: Effect on biotransformation of colchicine with different solutions

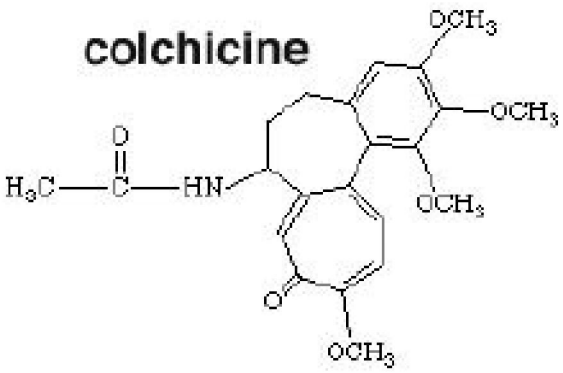

Figure 4: Chemical structure of colchicines

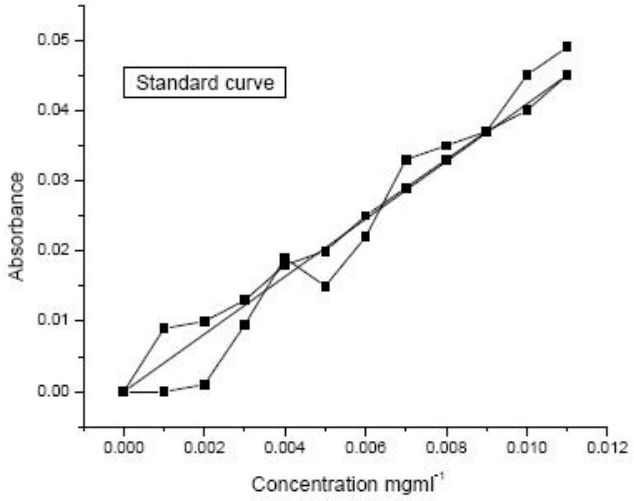

Figure 5: Effect of Bacillus megatarium on biotransformation of colchicines

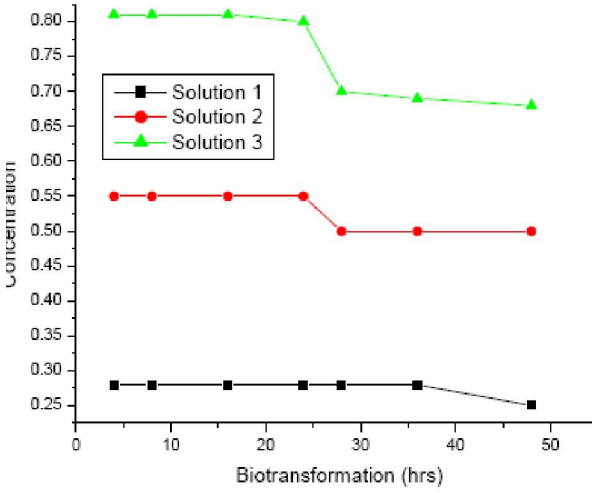

Figure 6 : Linear change of absorbance with respective change in concentration of the solution

TABLE 1 : Colchicine composed solutions

Soln. 1- 25ml sterile broth, 2ml culture, 12.5mg colchicines (0.5mg/ml)
Soln. 2-25ml ST broth, 2ml culture, 17.5 mg colchicines (0.7mg/ml)
Soln. 3-25ml ST broth, 2ml culture, 22.5 mg colchicines (0.9mg/ml)
Soln. 4-reference for 1 -25 ml ST broth, 12.5 mg colchicines.
Soln. 5-reference for 2 -25 ml ST broth, 17.5 mg colchicines.
Soln. 6-reference for 3 -25 ml ST broth, 22.5 mg colchicines.
Soln. 7-reference for 1 -25ml ST broth, 2 ml bacterial culture.
Then perform TLC after interval of time to check for Rf value.
Stationary phase: silica gel coated glass plate.
Mobile phase: acetone: ethyl acetate: water (5:4:1)
10% alcoholic sulphuric acid, act as indicator of compound spot.

Seven solutions were prepared to calibrate the standard curve. Quantity of colchicines was changed to know the sustainability of the strain bacillus megataruim and their growth of colonies.

TABLE 2 : LB-AGAR (Sterilization: 121°C20') - pH 7

*MATERIAL USED	QUANTITY
Triptone	10g/l
Yeast extracts	5g/l
NaCl	10g/l
Agar	15g/l

Liquid broth agar (LB agar) preparation

TABLE 3 : Broth ST (Sterilization: 121°C. 20') - pH 7

*MATERIAL USED	QUANTITY
Glucose	20g/l
Glycerol	10g/l
Peptone	15g/l
Yeast extracts	5g/l
NaCl	3g/l
NH_4Cl	3g/l
K_2HPO_4	8g/l
KH_2PO_4	3g/l
$MgSO_4.7H_2O$	0.5g/l

Sterile broth (ST broth) preparation

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