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Enhanced Production of Oxytetracycline from *Streptomyces rimosus* NCIM 2213 using Pretreated Sugarcane Molasses by Statistical Optimization

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Oxytetracycline (OTC) is a macrolide broad spectrum antibiotic produced widely by microbial fermentation using *Streptomyces Species*. In the present study, pretreated sugar cane molasses (SCM), an industrial crop by-product was used as main substrate for the production of OTC from *Streptomyces rimosus* NCIM 2213. Four different pretreatment methods and their combinations were applied to SCM to remove the heavy metals and toxins. Sulfuric acid treated molasses was found effective based on the cell mass and OTC yield. The enhanced production of OTC was achieved by statistical optimization using Central composite design with four chosen variables. From the second order polynomial equation, the optimum OTC concentration was attained by 537.73 mg/l from *S.rimosus* in sulfuric acid treated sugar cane molasses. To the best of our knowledge this is the highest yield of OTC obtained in submerged fermentation process. The Spectral characterization of purified OTC by UV, FTIR, ^{1}H NMR and ^{13}C NMR confirmed that the structure is to be homologous to the standard sample. Purified fraction was also showed remarkable antimicrobial activity against various Gram positive and Gram negative organisms.

Key words: Oxytetracycline (OTC), Molasses, Pretreatment, Streptomyces rimosus, Optimization, Central Composite Design (CCD).

Antibiotics are well known microbial secondary metabolites having significant commercial importance in treating human and veterinary infections; in agricultural and agri-food industry[1-4]. In fact 60% of the antibiotics produced have been commercially used in agricultural sector for the control of plant diseases, stimulation of amino acid fermentation and inhibition of material biodeterioration[5, 6]. *Streptomyces species* have been considered to be the most efficient and effective producer of diverse secondary metabolites[6-10] almost producing 66% of commercial antibiotics used worldwide[11]. This global range utilization of *Streptomyces sp* substantiates its importance in the production of antibiotics on an industrial scale[12-15].

Oxytetracycline (OTC) is a broad spectrum antibiotic mainly used to treat a wide range of infections like *Mycoplasma, Rickettsias, Trachoma, Amoebae, Balatidia* etc.[16-19]. Various strains of *Streptomyces sp*. especially *Streptomyces rimosus* are widely employed for the production of OTC at large scale for commercial purpose[12, 18, 20, 21]. Industrially antibiotics have been producing by both submerged fermentation and solid state fermentation process[22]. Fermentation medium usually represents almost 60% of the cost for a microbial fermentation[23]. Employing complex media for the production of microbial metabolites is not

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cost effective due to the usage of large quantities of expensive nutrients like as yeast extract, peptone, and salts. So, to achieve a cost effective fermentation process at high production yields it is a prerequisite to design an optimal cost-effective production medium. Therefore in many production processes the main substrate for the fermentation process, glucose has been replacing by cheap and complex carbon sources like molasses, starch, cellulose etc[24-25].

The present investigation was aimed to enhance the production of OTC from *Streptomyces rimosus* NCIM 2213 using an industrial crop waste, sugar cane molasses by submerged fermentation. A Central Composite Design (CCD) was used to get the optimum concentration of a series of selected medium ingredients that influence the OTC production[21,26]. The linear, square and interaction effect of the variables on the production of the OTC were studied. This statistical based optimization process involves three steps- design of experiments using CCD; estimating the mathematic model for the yield; and predicting the response and checking the adequacy of the model[27]. The present work also focuses on the extraction, purification and characterization of produced OTC.

MATERIALS AND METHODS

Microorganisms

Streptomyces rimosus NCIM 2213 was procured from National Chemical Laboratory, Pune, India; cultured and maintained on MGYP medium. All the other reference strains used in this study were procured from Microbial Type Culture Collection and Gene bank, Chandigarh, India, cultured and maintained on nutrient medium. Muller Hinton agar medium was used throughout the work for antimicrobial assay[15].

Chemicals

All chemicals and solutions used in this study were supplied by Merck (Germany), Sigma (USA) and Hi-media (India).

Molasses and its pretreatment

Sugar cane molasses (SCM) is a byproduct of the manufacture of sucrose from sugar cane. SCM containing 40% total sugars and 20% glucose with an initial pH of 7.2 was supplied by DECCAN Sugars, Nava Bharat Ventures, Samalkota, Andhra Pradesh, India. SCM was subjected to different pretreatment methods like Ferric cyanide, sulfuric acid, tricalcium phosphate and Hydrochloric acid treatments as well as their different combinations. Before and after the pretreatments SCM was analyzed for its total carbohydrate concentration by the phenol/sulfuric acid method[28] and then diluted with distilled water to an appropriate final carbohydrate concentration.

Sulfuric acid treatment: SCM pH was adjusted to 3.0 with concentrated H_2SO_4 and allowed to mix for 24 h; Tri calcium treatment: SCM was treated with 2% tricalcium phosphate and allowed to mix for 4 h; Ferric cyanide treatment: SCM was treated 0.6% of ammonium ferric cyanide and allowed to mix for 2 h; Hydrochloric acid treatment: SCM pH was adjusted to 3.0 with 0.1N HCl and allowed to mix for 24 h. All these treated solutions were subjected for centrifugation at 5,000×g for 15 min. The pH of the clear supernatants was adjusted to 7.0 and then used as the carbon source for the experiments[29].

Production medium for OTC and cultivation conditions

Batch shake flask experiments were carried out in 250-mL Erlenmeyer flasks with 100 ml of diluted sterile pretreated SCM by inoculating 5ml of 24 hr well grown culture broth of *Streptomyces rimosus* in a REMI orbital shaking incubator at 150 rpm for 4 days at 28^0C[26].

Purification and characterization of OTC

After centrifugation of fermented broth at 10,000 rpm for 20 minutes the pH of the clear supernatant was adjusted to 4.0 to increase the antibiotic solubility in the organic phase. Liquid–liquid extraction using ethyl acetate was carried out for thrice to extract OTC from the clarified fermented broth (1 part of supernatant and 4parts of ethyl acetate). The pH range 4-8 was used to shift the OTC between aqueous and organic phase. Calcium chloride (0.11 %) was used as a chelating agent. All the ethyl acetate fractions were concentrated in a Rota-vapor at 37^0C and subjected to silica column chromatography. The active compound obtained from the silica column was characterized using chemical and physical methods (UV, FTIR, and NMR).

Color reactions of purified compound with Con H_2SO_4, Con HCl and 2N NaOH were also recorded under both the visible light and UV light

to confirm the presence of OTC. Reagent mixture was also boiled for determine variability in color[20].

Ultraviolet (UV) spectrum of the purified compound in methanol was recorded with a Thermo Evolution 201 spectrophotometer at 200–400 nm. Infrared (IR) spectrum of the purified compound was recorded on FT-IR SPECTRUM RX-I spectrometer in the range of 400–4,000 cm-1 using KBr pellet technique. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra of the purified compound in deuterated water were conducted with 300 MHz BRUKER NM-4950 AVANCE II instrument [20, 26, 30].

Construction of Calibration curve for OTC from antimicrobial assay

Antimicrobial assay was performed by using disc diffusion method. Disc containing antimicrobial compound was placed on the Mueller Hinton agar plated with the test organism at standard set of conditions like medium volume, inoculum size, incubation temperature etc. After 18 hr incubation, the diameter of zone of inhibition was measured (mm) and OTC yield was computed form the Standard curve (mg/10ml)[20, 21, 31]

Standard Oxytetracycline procured from HIMEDIA laboratories, India was used for preparing standard curve and regression equation was developed, $Y = 0.1356X - 0.561$ ($R^2 = 0.963$), where, Y = log of concentration, X = (Zone of inhibition – Disc Size) cm. *E. coli* was used as a test organism for plotting standard and also determining unknown concentration[6, 31]

Optimization of the production medium by RSM using CCD

The significant media components were optimized using Response surface methodology (RSM), a statistical technique used for modeling and optimization of multiple variables. RSM uses the mathematical and statistical approaches to analyze the effect of preselected variables on the yield without having the prior knowledge about the relationship between the variables and response function. It involves design of experiments, development of model, evaluation of the effect of all factors and optimization of the desired response by reducing the number of required experiments[32]. In this study, Central Composite Design (CCD) was used to explore non-linear relationships between independent (Concentration of Ammonium sulfate, Calcium carbonate, Initial pH and inoculum size, mention our variables) and the dependent (OTC yield) variables[7, 18]. These relationships assist in selecting the concentrations of the medium components producing maximum product. To study the combined effect of these variables on the OTC production, 4 variables, 5 levels, 26 trail run was sketched according to CCD[27]. The application of RSM resulted in following regression equation, showing relationship between the predicted response and the coded levels of independent variables.

$$Y=\beta_0+\Sigma\beta_i X_i+\Sigma\beta_{ii} X_{ii}^2+\Sigma\beta_{ij} X_i X_j \quad ...(1)$$

Where Y is the predicted response, X is the coded levels of the independent variables, β_0 is the offset term, β_{ii} is the i^{th} squared coefficient and β_{ij} is the ij^{th} interaction coefficient. These experiments were performed thrice and the average values were considered for the analysis. All the other experiments in this study were conducted thrice and reported the average values with the standard error.

RESULTS AND DISCUSSION

Effect of pretreatment of SCM on the production of OTC

Molasses, an industrial by-product of sugar cane widely employed as a fermentative substrate for the production of microbial metabolites due to its high sucrose and other nutrient contents, low cost and ready availability, and ease of storage. To reduce the production cost of oxytetracycline, pretreated sugarcane molasses (SCM) was chosen in the present study as the substrate for the submerged fermentation by *S.rimosus* NCIM 2213.

Total sugar concentration and reducing sugar concentration was almost same for SCM before and after pretreatments hence carbohydrate content was not affected by any of the pretreatments performed. All the pretreated SCM samples were screened for the better production of OTC. The produced OTC was purified and quantified from the calibration curve for standard OTC. Highest yields of OTC were observed with the Sulfuric acid treated SCM (Figure 1). So, sulfuric acid treated SCM was considered as a substrate throughout the study for enhanced production of OTC from *S.rimosus*. The concentration of sulfuric

acid in the pretreatment was further optimized by adjusting SCM pH from 2.0- 5.0 with sulfuric acid, OTC production was carried at standard set of conditions and maximum OTC yield was observed at pH 3.0 (Figure 2).

Physico chemical characterization of produced and purified OTC

The results of chemical characterization of OTC based on the color formation were given in Table 1. Tetracyclines (TC, OTC, and CTC) will react differently with various chemical treatments

Table 1. Chemical tests for standard and purified OTC

S. No	Reagent	Color appearance under Normal light		Color appearance under UV illumination	
		Standard	Pods	Standard	PODS
1	Con. H_2So_4	Reddish yellow	Red	Yellowish green	Black
2	Con. HCL (UB)	Greenish Yellow	Dark Yellow	Yellow	Yellowish green
3	Con. HCL (B)	Turbid yellow	Turbid Yellow	Slight Turbid	Highly Turbid
4	2N NaOH (UB)	Colorless	Yellow	Yellow	Yellow
5	2N NaOH (B)	Light Yellow	Dark Yellow	Light yellow	Dark yellow

*UB: Unboiled; B: Boiled

Table 2. Central Composite Design of the variables in real units for the response of OTC yield along with its predicted & observed values

Run no	Calcium carbonate (g/l)	Ammonium sulfate (g/l)	Inoculum size (% V/V)	pH	Yield (mg/l)	
					Experimental	Predicted
1	10	80	8	5.5	225.4084	172.398
2	10	10	7	7.5	359.3106	400.388
3	0.2	10	8	7.5	141.4067	170.865
4	5	50	4	6.5	1066.522	1045.780
5	5	90	10	6.5	263.3116	329.896
6	5	50	10	6.5	490.3092	555.324
7	0.2	80	7	5.5	103.6263	161.478
8	5	50	10	5	121.0515	136.851
9	0.2	80	8	5.5	225.4084	152.780
10	0.2	80	8	7.5	225.4084	164.668
11	0.2	10	7	5.5	419.73	411.275
12	5	8	10	6.5	141.4067	140.656
13	0.2	10	8	5.5	225.4084	247.089
14	10	80	8	7.5	419.73	264.748
15	5	50	10	6.5	572.7565	555.324
16	10	10	8	7.5	307.5884	257.186
17	10	10	7	5.5	359.3106	401.951
18	0.1	50	10	6.5	572.7565	540.646
19	10	80	7	7.5	141.4067	252.463
20	5	50	14	6.5	912.9984	898.100
21	20	50	10	6.5	912.9984	928.519
22	5	50	10	8	141.4067	174.555
23	0.2	80	7	7.5	121.0515	167.567
24	10	80	7	5.5	141.4067	165.913
25	0.2	10	7	7.5	359.3106	329.250
26	10	10	8	5.5	307.5884	252.949

Table 3. Regression analysis of the central composite design.

Factor	SS	MS	F	P
X_1	9052	9052.0	1.27951	0.282048
X_1^2	10222	10222.0	1.44489	0.254588
X_2	3906	3905.6	0.55207	0.473044
X_2^2	215279	215279.2	30.42997	0.000182
X_3	115715	115715.4	16.35652	0.001934
X_3^2	181523	181523.0	25.65849	0.000363
X_4	529	528.9	0.07477	0.789586
X_4^2	238457	238457.1	33.70620	0.000118
$X_1 \times X_2$	189	189.4	0.02677	0.872998
$X_1 \times X_3$	767	767.0	0.10842	0.748134
$X_1 \times X_4$	6474	6474.2	0.91514	0.359306
$X_2 \times X_3$	110313	110312.8	15.59284	0.002277
$X_2 \times X_4$	7769	7769.2	1.09818	0.317145
$X_3 \times X_4$	217	217.4	0.03073	0.864024

*Highlighted values are more significant

(H_2SO_4, HCL, 2N NaOH) and gives different color reactions at conditions like boiling and illumination. The purified compound was given the same color appearances with standard OTC under both illuminations (UV & Visible) with all the tested chemical methods.

The UV spectrum (Fig. 3a) of the purified OTC in methanol had shown two characteristic peaks at 354 and 277 nm resembling the presence of carbonyl and aromatic group respectively and confirms the presence of tetracycline[33].

The FT-IR spectra of purified and standard OTC was referenced according to Singh et al., 2013, showed characteristic bands at 1514 (C=O), 1420 (C-N stretching of tertiary amine or dimethyl amine), 1206 (C-O of secondary alcohol)and 986 (C=C stretching of alkene / C-H of alkene) respectively (Figure 3b). The wavelength region 1300-1,700/cm is reported to be fingerprint of molecule because it allows the identification of major chemical groups in tetracycline.

Further confirmation of OTC was performed using ^{13}C NMR and ^{1}H NMR analysis. ^{13}C NMR (Figure 3c&3d) and ^{1}H NMR (Figure 3e&3f) were shown to have peaks comparable with the standard OTC and hence confirmed the presence of oxytetracycline in the extracted fraction. ^{13}C NMR spectrums shown resonances for C

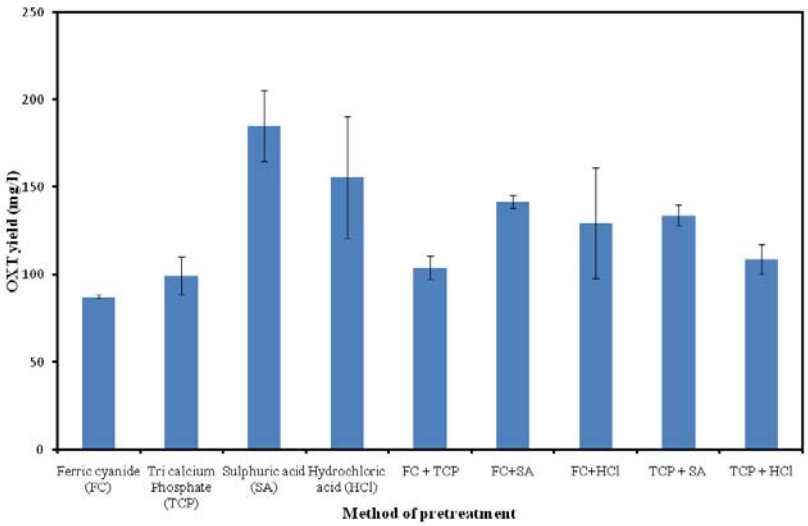

Fig. 1. Effect of various chemical pretreatments on Molasses for OTC production

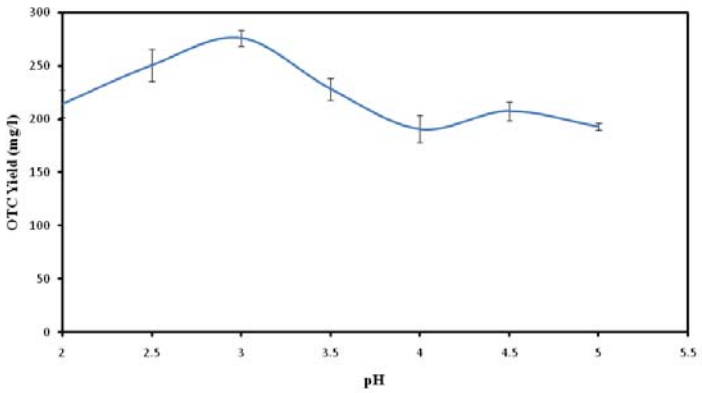

Fig. 2. Optimization of sulfuric acid pretreatment of molasses for OTC production

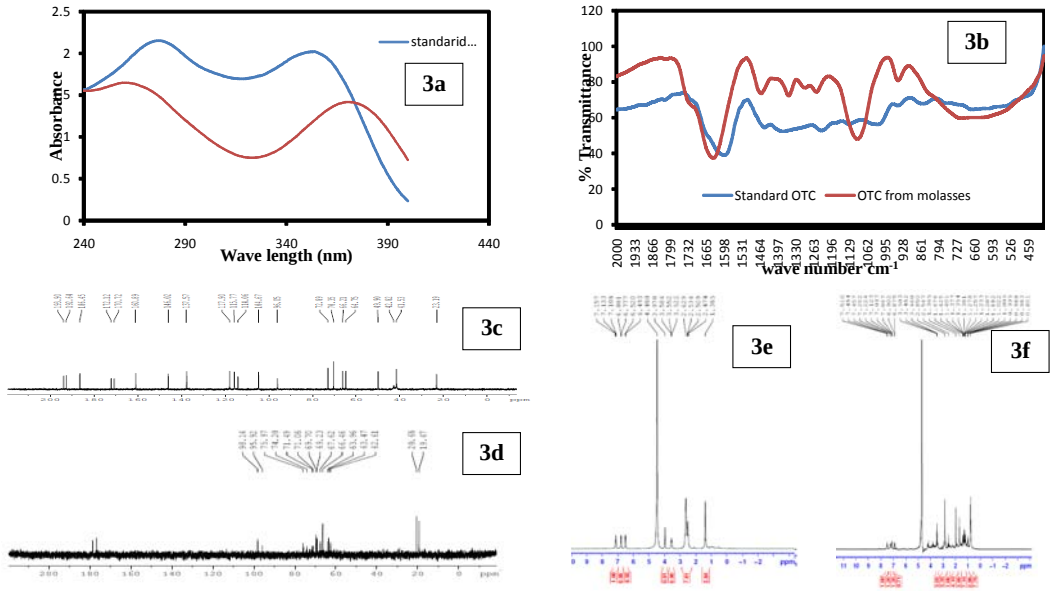

Fig. 3. UV spectra for standard OTC and Purified OTC

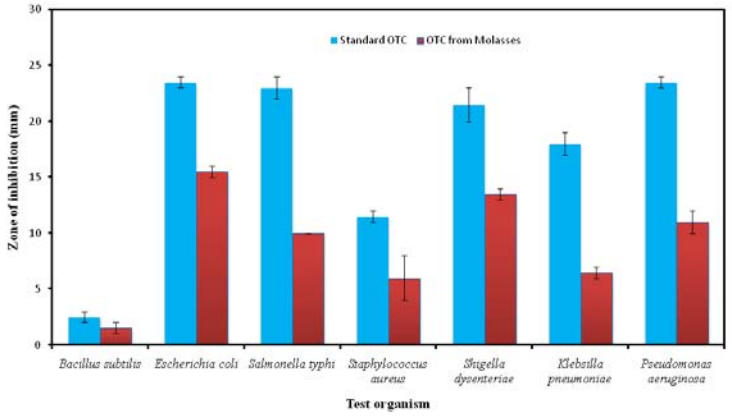

Fig. 4. FT-IR spectra for standard OTC and Purified OTC

signals in the range 193 to 16 ppm for both standard and purified OTC. Functional group based on peaks for purified OTC; 193.90 - 186.45, >C=O; 172.12, H_2N>C=O; 170.72 – 172.12, C=C; 172.89 – 186.45, C=C of aliphatic; 104.67 – 149.02, Ar- H; 42.82 – 72.89, Cyclohexane; 23.19 – 70.35, - CH_3. The ^1H NMR spectrum was showing identical pattern peaks of various resonances, triplets peaks at 7.15 - 7.108 ppm (Standard OTC) and 7.0-7.5(purified OTC) were indicating CH of aromatic group. Peaks at 1.385(Standard OTC) and 1.301 (purified OTC) indicates CH_3-C-OH of oxytetracycline. CH_3 of aliphatic were noted by peaks in 2.474 – 2.629 (Standard OTC) and 1.965 – 2.850 (purified OTC)[20, 33]. Based on both the physical and chemical characterization techniques the produced and purified compound was confirmed as oxytetracycline

Antimicrobial activity of purified OTC

Purified OTC had shown relatively good antimicrobial activity against various selected Gram positive and Gram negative organisms; *Bacillus subtilus, Escherichia coli, Klebsiella pnemoniae, Shigella dysenteriae, Salmonella typhi, Staphylococcus aureus* and *Pseudomonas aeruginosa* compared to the standard OTC (Fig. 4). The tested pathogens were sensitive to purified compound and further no growth was observed. This clearly describes the bactericidal activity of purified OTC.

Optimization of oxytetracycline production by RSM

The effect of various parameters on the production of OTC like the initial substrate (pretreated SCM) concentration, calcium carbonate concentration, influence of environmental conditions (Initial pH, Incubation time and temperature, Inoculum size and agitation speed) and supplementary nutritional conditions (Carbon source like sucrose, maltose, lactose, Glucose and fructose; nitrogen sources (both organic and inorganic) were studied (data not shown). The most significant variables, Calcium carbonate, Ammonium sulfate, Inoculum size and pH were selected for further optimization by RSM.

CCD was employed to study the interactive effect among these significant variables, Calcium carbonate (X_1), Ammonium sulfate (X_2), Inoculum size (X_3) and pH (X_4); each at five levels on the production of OTC from SCM and also to determine their optimal levels for maximum OTC yield. Table 2 shows the design of experiments in real units using CCD with both experimental and predicted values. Good correlation between the observed and predicted values was observed.

The statistical significance of the model was checked by F-test, and ANOVA for the response surface quadratic model are summarized in Table 3 and having $p<0.0005$ at 95% significance level square interactions of ammonium sulphate, inoculums size and pH were found to be significant. This represents the need of readily available nitrogen source and maintenance of pH throughout the stipulated fermentation. ANOVA of the quadratic regression model were significant with Fisher's F-test, with high F- value and low P value. The coefficient of regression (R^2) is the ratio of sum of squares due to regression and total sum of squares and can be interpreted as the proportion of variability in the data explained by ANOVA. The R^2 value of 0.95505 shows that more than 95% of the experimental data can be explained by the model[34]. The adjusted R^2 corrects the R^2 value for the sample size and number of terms involved in the model. High adjusted R^2 of 0.89784 implies a high correlation between the experimental and predicted yields.

The significant regression coefficients shows the model to be as follows

$$Y=567.808 – 231.685\ X_2^2 + 37.673\ X_3^2 – 177.609\ X_4^2$$

Where Y is the response i.e, OTC yields. The critical values for the significant variables were predicted through RSM as 1.54871 g/l of Calcium carbonate; 51.84731 g/l of ammonium sulphate; 9.47862 % (V/V) of inoculums size; and pH 6.49451. The predicted optimum yield using these critical values is 537.7238 mg/l. To the best of our knowledge this is the highest yield of OTC from *S.rimosus.*

CONCLUSION

SCM the byproduct of sugar cane was successfully employed as fermentative substrate for the production of OTC, an important class of antibiotics useful for the health care for human and veterinary and agro-poultry industry. The highest OTC yield was achieved when compare with the literature; 537.73 mg/l at the optimized conditions. This work once again proves that the

molasses is one of the best medium for the production of microbial metabolites. But the molasses consists of impurities like heavy metals and toxins that interfere the microbial metabolism and subsequently reduces the product yield. Of course pretreatment process will remove most of these but before making this into commercial platform one has to overcome the hurdles with the crude/raw molasses pretreatment.

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