Production and Statistical Optimization of Oxytetracycline from *Streptomyces rimosus* NCIM 2213 using a New Cellulosic Substrate, *Prosopis juliflora*

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Prosopis juliflora is a drought-resistant evergreen spiny tree that grows in semi-arid and arid tracts of tropical and sub-tropical regions of the world. Dry pods of *P. juliflora* are a rich source of carbon (40% total sugar) and nitrogen (15% of total nitrogen) and so can be considered as a good substrate for the microbial growth. The present study was mainly focused on the utilization of these pods for the production and statistical optimization of oxytetracycline (OTC) from *Streptomyces rimosus* NCIM 2213 under SSF. The spectral characterization and chemical color reactions of purified OTC by UV, FTIR, 1H NMR, 13C NMR, and HPLC revealed that the structure was homologous to a standard sample. A central composite design with 26 trails yielded the following critical values of supplements to be added to the dry pods: maltose (0.125 g/gds), Inoculum size (0.617 mL/gds), $CaCO_3$ (0.0026 g/gds), and moisture content (74.87%) with the maximum OTC yield 5.02 mg/gds.

Keywords: Oxytetracycline (OTC); Prosopis juliflora; Solid state fermentation (SSF); Central composite design (CCD); Purification; Characterization

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INTRODUCTION

Prosopis juliflora is a shrub of semi-arid and tropical part of the world and is spreading due to its non-utilization by both humans and animals. An average plant yields annually 10 to 50 kg of pods/ tree within 3 years of its seeding. In general, these can be collected from May-June and September-October. The total productivity of pods has been estimated to be about 2 to 4 million metric tons worldwide. On a dry matter basis, these ripened pods consist of 12% crude protein, 15% free sugar, and a moderate level of digestible crude protein (7% DCP). The pods contain tannins below the toxic concentration of animals (Sawal *et al.* 2004). Indian pods consist of 16.5 to 7.6% crude proteins (Talpada *et al.* 1987), 28 to 19% fiber content (Anon 1943; Talpada *et al.* 1988), and 46.3 to 61.6% nitrogen-free extract (Anon 1943; Talpada *et al.* 1987). Lack of the knowledge about nutritional value of pods and neuro-toxic side effects on pods consuming cattle has restricted their utilization as a fodder (Tabosa *et al.* 2006) (Saykhedkar and Singhal 2004).

Tetracyclines are a family of antibiotics that share the same four cyclic ring and include tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC), and dameclocycline (DC) (Sevilla-Santos *et al.* 1987; Pickens and Tang 2010). It acts against Gram positive bacteria, Gram negative bacteria, parasites, and some fungal pathogens (Asagbra *et al.* 2005). Bacteriostatic and bactericidal activity of tetracycline's family has extended its application in veterinary science for animal disease, agriculture to control plant

pathogens (McManus and Stockwell 2001; McManus *et al.* 2002), and medical fields for treating infections, *etc*. (Nikolakopoulou *et al.* 2005).

Solid-state fermentation (SSF) processes are of particular interest due to their high product yield, low costs due to the efficient utilization, value-addition of widely available wastes, and eco-friendly behavior in disposal (Subramaniyam and Vimala 2012). The production of OTC by SSF has been studied using sweet potatoes, potato residues (Yang and Swei 1996), corn cobs (Yang and Swei 1996; Okorie and Asagbra 2008), cassava peels, peanut shells (Asagbra *et al.* 2005), sawdust, and rice hulls (Saykhedkar and Singhal 2004).

Statistical optimization has been used to reduce the number of trials and cost of experiments by proper design and analysis to find out the optimum concentrations of a series of medium ingredients that are contribute for the maximum product formation (Zeng *et al.* 2006; Mao *et al.* 2007; Kumar *et al.* 2014). Response surface methodology is used for the study of linear, square, and interaction effect of the variables on the production of bio-chemicals (Lotfy 2007; Wang *et al.* 2008, 2013; Yu *et al.* 2008; Singh *et al.* 2012; Uppuluri *et al.* 2013b).

In the present study, the use of lignocellulosic weed *Prosopis juliflora* as a potential substrate for the fermentation (SSF) was exploited. OTC production was carried out using the pods of *Prosopis juliflora* from *Streptomyces rimosus* NCIM 2213 by SSF. RSM-based central composite design was used to optimize cultural conditions *viz*., the concentration of maltose, $CaCO_3$, inoculum size, and moisture content for the maximum OTC production. Further, spectral characterization of purified OTC by UV, FTIR, ^{1}H NMR, and ^{13}C NMR was performed in addition to the HPLC analysis. To the best of our knowledge, this is the first report on the utilization of pods of *Prosopis juliflora* as a solid state fermentation substrate.

EXPERIMENTAL

Materials

Microorganisms and media

Streptomyces rimosus NCIM 2213 was procured from National Chemical Laboratory, Pune, India. It was 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. Test strains was cultured and maintained on nutrient medium and Muller Hinton agar medium was used for antimicrobial assay.

Pods collection

Prosopis juliflora pods were collected outside the SASTRA university campus, Thanjavur, Tamil Nadu, India. Substrates were collected from the same region throughout the work to avoid bias due to change in nutritional content. Carbon sources including cellulose (Kulić and Radojičić 2011), total organic carbon, reducing sugar as glucose, lignin, fiber, and moisture content were determined using standard assays.

Production of OTC by SSF

Prosopis juliflora pods were washed first with tap water followed by distilled water to remove the adhered surface dust particles. Then a bleaching operation was carried out by immersing them in hot water (75 to 80 ^{o}C) for 20 min followed by oven drying at 45 ^{o}C. The dried material was grounded to course powder using a mixer grinder. Initially 10

g of dried ground pods powder was taken in a 250 mL Erlenmeyer flask and to which a predetermined quantity of phosphate buffer, pH 7.4 was added, mixed thoroughly, and then sterilized at 121 ºC, 1.06 kg/cm^2 for 15 min. The sterilized medium was inoculated with 2 mL of inoculum containing 10^7 spores per mL of *Streptomyces rimosus* NCIM 2213 and incubated for 9 days at 28 ºC. The media were mixed regularly for uniform distribution of organisms.

Purification and Characterization of OTC

Fermented matter was mixed with phosphate buffer, pH 7.4 (1:4 w/v) by stirring on a magnetic stirrer for 30 min at room temperature. The slurry was then centrifuged at 10,000 × g for 10 min at 4 ºC to remove the insoluble matter. The clear supernatant was used for purification and estimation of OTC yield. Liquid–liquid extraction using ethyl acetate along with 0.1% calcium chloride as chelating agent was performed with the supernatant to extract the OTC, at pH 4 to 8. All the ethyl acetate fractions were concentrated in a Rota-vapor at 37 ºC and subjected to silica column chromatography (Sevilla-Santos *et al.* 1987; Singh *et al.* 2013).

The ultraviolet (UV) spectrum of the purified compound in methanol was recorded with a Thermo Evolution 201 spectrophotometer at 200 to 400 nm. The infrared (IR) spectrum of the purified compound was recorded on an FT-IR SPECTRUM RX-I spectrometer in the range of 400 to 4,000 cm^{-1} using the KBr pellet technique. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra of the purified compound in deuterated water (D_2O) were conducted with a 300 MHz BRUKER NM-4950 AVANCE II instrument (Chen *et al.* 2012; Singh *et al.* 2012)

Color reactions of the purified compound with concentrated H_2SO_4, concentrated HCl, and 2N NaOH were also recorded under both the visible light and UV light to confirm the presence of OTC. The reagent mixture was also boiled for determine the variability in color (Patrocinio *et al.* 1987; Chen *et al.* 2012).

Chromatographic analysis was performed with PEAK high performance liquid chromatography having LC-P7000 isocratic pump, equipped with PEAK LC-UV7000 variable wavelength detector. UV detection was made at 254 nm with column temperature of 30 ºC. The flow rate was set to 0.6 mLmin^{-1}, and injections of 20 µL were made. Acetonitrile and deionised water (80% and 20%) were used as the mobile phase (Singh *et al.* 2012).

Construction of Calibration Curve for OTC from Antimicrobial Assay

An antimicrobial assay was performed by using the disc diffusion method. A disc containing an antimicrobial compound was placed on the Muller Hinton agar plated with the test organism with a standard set of conditions including medium volume, inoculum size, and incubation temperature. After 18 h of incubation, the diameter of zone of inhibition was measured (mm), and OTC yield was computed form the standard curve (Yang and Swei 1996; Asagbra *et al.* 2005).

Standard oxytetracycline was used for preparing the standard curve, and a regression equation was developed having the form $Y = mX + C$, where Y is the log of concentration, and X is the zone of inhibition minus the disc size) (cm). *E. coli* was used as a test organism for plotting the standard and also determining the unknown concentration (Yang and Ling 1989; Asagbra *et al.* 2005).

Optimization of Selected Medium Components by Central Composite Design

The central composite design (CCD) known as Box-Behnken is the most accepted and widely used design to study the interaction effect of the medium components (Adinarayana and Ellaiah 2002). The CCD is a statistical experimental design where each numeric factor is varied at 5 levels (-2, -1, 0, +1, +2). Four significant variables (maltose concentration, $CaCO_3$ concentration, initial moisture content, and inoculum size) were chosen for the experiment. The experimental design was performed using coded values to avoid bias in the experiments and the effects of linear, nonlinear interactions were studied using response surface methodology. A sum of 26 experiments was performed in triplicates, and the average value was considered for obtaining the polynomial equation (1) to predict optimum value:

$$Y = \mu_0 + \sum \mu_i Z_i + \sum \mu_j Z_{i2} + \sum \mu_{ij} Z_i Z_j \quad (1)$$

In Eq. 1, Y is the predicted yield, μ_0 is the linear interaction coefficient, μ_i is the quadratic interaction coefficient, μ_{ij} is the interaction coefficient, and Z_n terms are variables. The design and analysis of experiments were carried out using "STATISTICA 8.0" software, evaluation version (Mao *et al.* 2007; Kammoun *et al.* 2008; Yong *et al.* 2011; Kumar *et al.* 2014).

Antimicrobial Assay against Different Pathogens

The potency of OTC produced and purified from *S. rimosus* NCIM 2213 using *P. juliflora* pods was tested against various pathogens. Reference strains including *Bacillus subtilis* (MTCC 441), *Escherichia coli* (MTCC 723), *Salmonella typhi* (MTCC 531), *Staphylococcus aureus* (MTCC 3160), *Shigella dysenteriae* ATCC 23513, *Klebsilla pneumonia* (MTCC 1667), and *Pseudomonas aeruginosa* (MTCC 2488) were employed (Singh *et al.* 2013). An antimicrobial assay was triplicated according to the above procedure, and the zone of inhibition was reported in mm along with SE.

RESULTS AND DISCUSSION

Chemical Composition of Pods

The detailed chemical composition of dried pods of *P. julioflora* is given in Table 1. This is the first report on the chemical composition of *P. julioflora* pods. Cellulose was found to be the major principle ingredient in pods. Cellulosic feed stocks and agricultural waste can be good fermentative substrates for the production of bio-chemicals, especially for secondary metabolites.

Table 1. Composition of Dried Pods of *Prosopis juliflora*

S. No	Component of *P. julioflora* pods	Quantity (g/g) on dry wt. basis
1	Cellulose	0.39 ± 0.004
2	Lignin	0.15 ± 0.0004
3	Total organic carbon	0.06 ± 0.0006
4	Total reducing sugar	0.009± 0.00015
5	Total fiber content	0.36± 0.0059
6	Moisture content	5.78 %

Total reducing sugar, total organic carbon, and fiber content results were in good agreement with the other species of *Prosopis* Pods (Alcedo 1988; Pasiecznik and Felker 2001; Sawal *et al.* 2004; Mabrouk *et al.* 2008).

Usage of agricultural and forestry residues as fermentative substrates for the production of value-added products including antibiotics under SSF is being widely practiced. Oxytetracycline can be easily produced from *Streptomyces rimosus* grown on a high cellulose containing medium by SSF. In fact, the oxytetracycline produced by SSF using corn cobs was more stable than that produced by Submerged fermentation (SmF) (Yang and Swei 1996). Peanut shells, corn cob, corn pomace, and cassava peels have been used as substrates for SSF to produce tetracyclines from *S. rimosus* (Asagbra *et al.* 2005)

Large quantities of *P. julioflora* waste have been generated and are spreading all over the world due to the unpalatability of leaves and pods, such that animals do not digest its seed. To overcome the environmental pollution problems associated with the conventional disposal methods of handling, this waste can be used as support-substrates in SSF to produce industrially relevant metabolites such as antibiotics with a great economical advantage.

Production of Oxytetracycline by SSF

The inoculated pods were observed for their time-dependent oxytetracycline production in batch flasks from the 24th hour to the 300th hour. OTC production was observed from the 96th hour, 0.2 mg OTC/gds and the maximum yield was found at the 216th hour, 0.44 mg OTC/gds. After that, a sudden fall of OTC yield was observed from 0.44 to 0.28 mg OTC/gds. This may be due to the autolysis of *Streptomyces rimosus*. A number of factors such as nutrient depletion, sudden change of environmental conditions, or intolerable limits of secondary metabolites may contribute for the autolysis of a microorganism and hence subsequent reduction of the metabolites. The action of produced antibiotic on the well-grown microorganism also may lead to the decrease in the already formed antibiotic (Okorie and Asagbra 2008; Rodríguez-Jasso *et al.* 2013).

Physicochemical Characterization of Produced and Purified OTC

The UV spectra of purified OTC showed two characteristic peaks at 349 nm and 277 nm, whereas the standard OTC at 349 nm and 274 nm indicated the presence of a carbonyl group and an aromatic group, respectively (results not shown) (Ruiz Medina *et al.* 2000; Singh *et al.* 2013). FT-IR spectra of purified and standard OTC showed the characteristic bands at 3465 cm^{-1}(O–H), 3457, 3441 cm^{-1} (N–H), 1597.36 cm^{-1} (C = O), and 1403.46 cm^{-1} (= C–N) (results not shown) (Ruiz Medina *et al.* 2000; Singh *et al.* 2013). The wavelength region 1300 to 1,700 cm^{-1} is reported to be fingerprint of molecule because it allows the identification of major chemical groups in tetracycline.

Further confirmation of OTC was obtained using ^{13}C NMR and ^{1}H NMR analyses. The ^{1}H NMR spectrum showed identical pattern peaks of various resonances, triplets peaks at 7.15 to 7.108 ppm (Standard OTC) and 7.407 to 7.353 (purified OTC) were indicating CH of aromatic group. Peaks at 1.385 (Standard OTC) and 1.2993 (purified OTC) indicate CH_3-C-OH of oxytetracycline. CH_3 of aliphatic were noted by peaks in the range 2.474 to 2.629 (Standard OTC) and 2.525 to 2.7(purified OTC) (Chen *et al.* 2012; Singh *et al.* 2013). ^{1}HNMR spectra for both standard OTC and purified OTC are shown in Fig. 1. ^{13}C NMR spectra in Fig. 2 show resonances for C signals in the range 193 to 16 ppm for both standard and purified OTC. Functional groups can be identified based on peaks 193.90 to 192.58

(carbonyl of aliphatic ring), 160.89 to 186.45(C=C), 72.89 to 96.05 (1-ethylene), 104.67 to 146.02 (phenyl group), and 42.82 to 73.3 (cyclohexane).

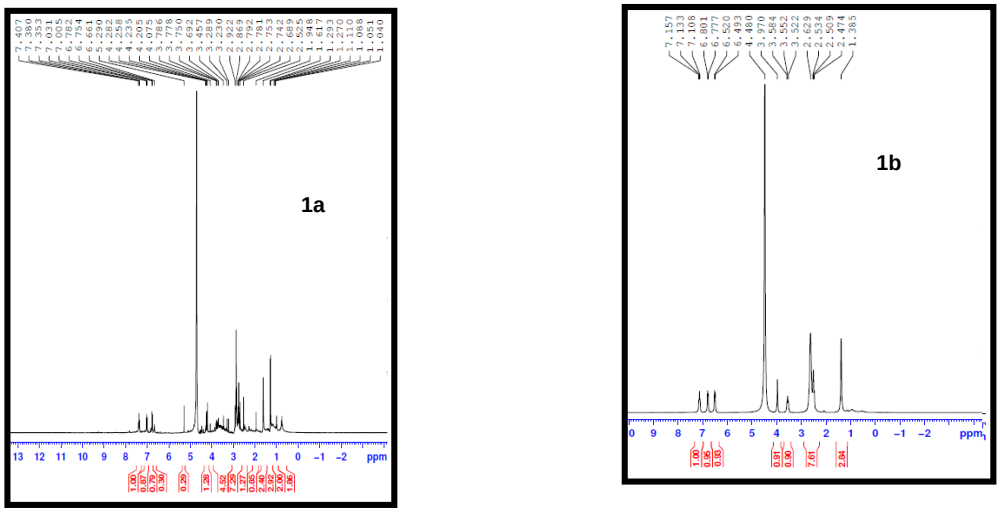

Fig. 1. ^{1}H NMR spectra for a) purified OTC b) standard OTC

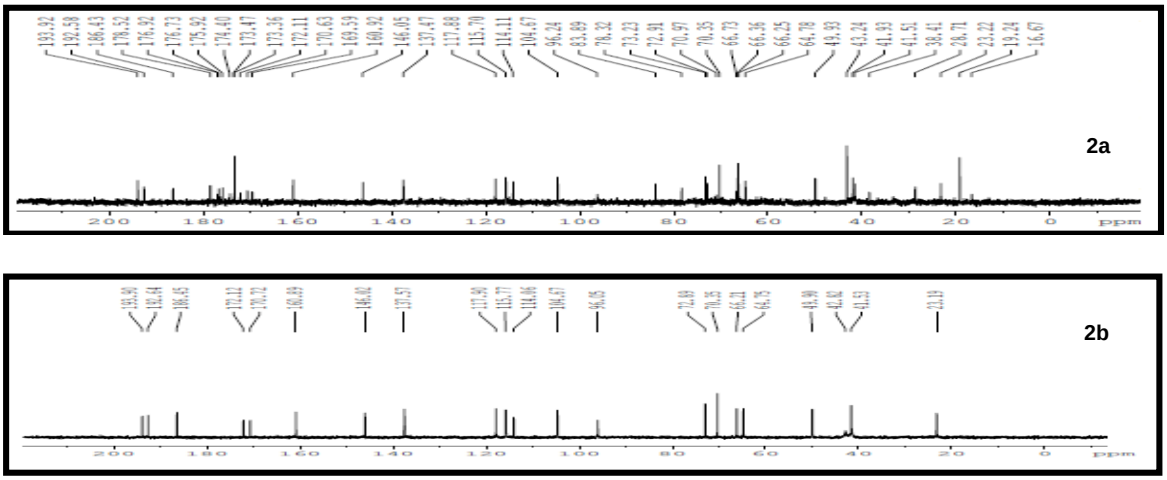

Fig. 2. ^{13}C NMR spectra for a) purified OTC b) standard OTC

Further, the produced and extracted yellow-colored compound chromatogram (Fig. 3) by HPLC showed a single peak with the retention time of 6.16 min. The same was observed with the standard OTC, confirming that the produced and extracted compound contains only one product and that it resembles the standard OTC.

The results of chemical characterization of OTC based on the color formation are given in Table 2. Tetracyclines (TC, OTC, CTC) will give different color reactions for various chemical treatments (H_2SO_4, HCl, 2 N NaOH) at conditions such as boiling and with illumination. The purified compound gave the same color appearances as the standard OTC and with literature reports under both illuminations (UV and Visible) with all the tested chemical methods (Sevilla-Santos *et al.* 1987; Singh *et al.* 2013). Based on both the physical and chemical characterization techniques, the produced and purified compound was confirmed as oxytetracycline.

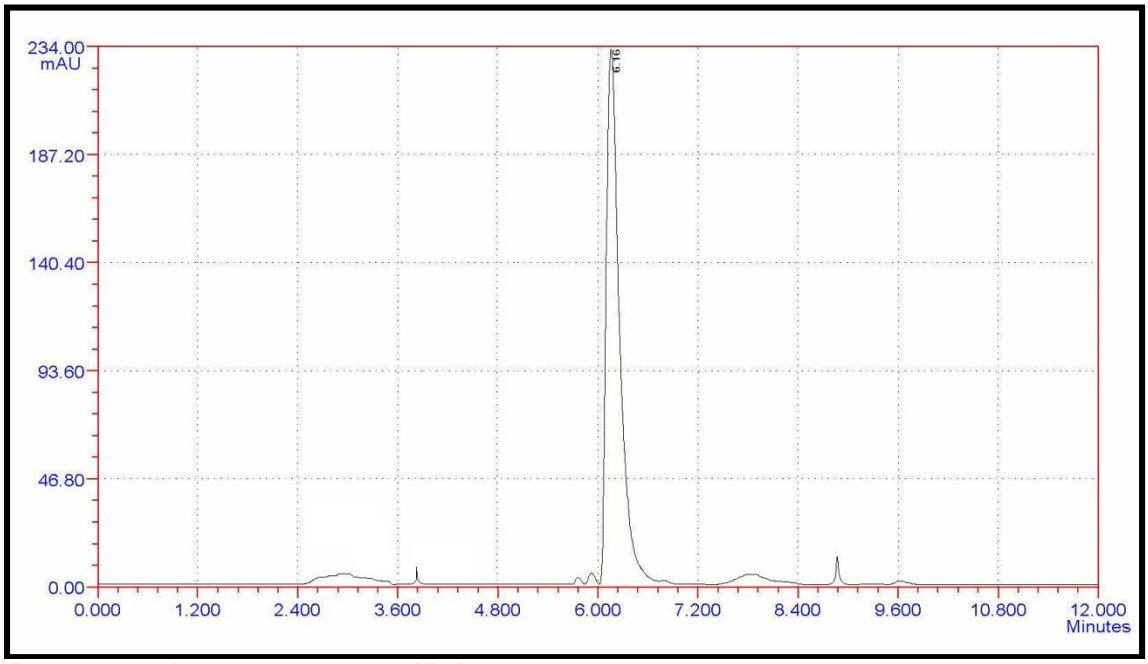

Fig. 3. HPLC chromatogram for purified OTC

Table 2. Chemical Tests for Standard and Purified OTC

S.No	Reagent	Color appearance under Normal light		Color appearance under UV illumination	
		Standard OTC	OTC from **Pods**	**Standard** OTC	OTC from **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

Calibration Curve from Antimicrobial Assay

A calibration curve was plotted between the log of concentration and the zone of inhibition (against *E. coli*) for the range of 2 µg to 30 µg under the standard set of conditions (Fig. 4). A linear equation was developed and used for the computing OTC yield in mg/mL throughout the study; $Y = 0.1427X - 0.647$, with $R^2 = 0.9891$ (Yang *et al.* 1996; Yang and Ling 1989; Okorie and Asagbra 2008; Okorie *et al.* 2008).

Statistical Optimization

Preliminary studies involving factors such as the influence of environmental conditions (initial pH, incubation time, and temperature), nutritional conditions (carbon sources such as sucrose, maltose, lactose, glucose, and fructose, nitrogen sources (both organic and inorganic), and inoculum size were conducted. The four most significant variables were chosen for further optimization by CCD for enhanced production of OTC from *S. rimosus* using dry pods.

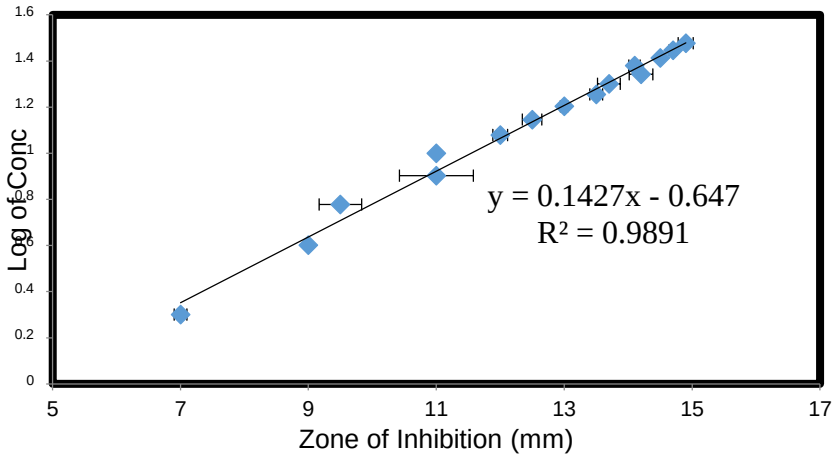

Fig. 4. Calibration curve for OTC from antimicrobial assay

The selected significant variables, maltose concentration, $CaCO_3$ concentration, inoculum size, and moisture (%) were optimized for maximum production of oxytetracycline by RSM (Kumar *et al.* 2014). The CCD experimental model was applied, leading to a second order polynomial equation for determining the optimized value (Zeng *et al.* 2006). Table 3 shows the Central Composite Design of the variables in real units for

Table 3. Central Composite Design of the Variables in Real Units for the Response of OTC Yield with Its Predicted and Observed Values

RUN NO	Maltose (g/10g)	Inoculum (ml/10g)	CaCo3 (g/10g)	Moisture (%)	Observed (mg/10g)	Predicted	Residual
1	1.5	8	0.025	30	16.73	22.96	-6.22
2	1.5	1	0.01	100	8.67	6.98	1.69
3	0.1	1	0.025	100	5.01	5.34	-0.32
4	0.8	4.5	0.001	65	23.25	25.10	-1.85
5	0.8	9	0.018	65	36.03	33.55	2.48
6	0.8	4.5	0.018	65	50.04	45.30	4.74
7	0.1	8	0.01	30	6.24	6.97	-0.73
8	0.8	4.5	0.018	10	8.67	8.44	0.22
9	0.1	8	0.025	30	16.73	15.90	0.83
10	0.1	8	0.025	100	23.25	24.60	-1.34
11	0.1	1	0.01	30	6.24	7.32	-1.08
12	0.8	0.5	0.018	65	12.05	20.21	-8.16
13	0.1	1	0.025	30	5.59	5.13	0.46
14	1.5	8	0.025	100	44.85	41.10	3.75
15	0.8	4.5	0.018	65	50.04	45.30	4.74
16	1.5	1	0.025	100	12.05	12.20	-0.15
17	1.5	1	0.01	30	4.49	4.28	0.20
18	0.08	4.5	0.018	65	36.03	37.57	-1.53
19	1.5	8	0.01	100	23.25	24.74	-1.49
20	0.8	4.5	0.03	65	44.85	43.98	0.87
21	1.6	4.5	0.018	65	40.12	43.89	-3.77
22	0.8	4.5	0.018	110	23.25	27.55	-4.30
23	0.1	8	0.01	100	7.77	8.69	-0.92
24	1.5	8	0.01	30	16.73	13.57	3.16
25	0.1	1	0.01	100	5.59	0.56	5.02
26	1.5	1	0.025	30	6.24	2.54	3.70

the response of OTC yield (average value) along with its predicted and observed values. Regression analysis of the central composite design is given in Table 4.

Equation 2 was framed by considering the only significant variables ($p<0.005$) on the OTC production; hence insignificant variables, $p>0.005$, were omitted in the equation (Gharibzahedi *et al.* 2012).

$$Y = 45.0979 + 3.4272\ Z_i + 7.1960\ Z_j + 3.5876\ Z_k + 2.7883\ Z_l - 12.834\ Z_{2j} - 2.5149\ Z_{2k} - 13.0642\ Z_{2l} + 2.1885\ Z_j\ Z_k \quad (2)$$

In Eq. 2, Y is the OTC yield with respect Z_n independent variables, for the equation based on linear, quadratic, and interaction coefficients of the variables.

Accuracy and fitness of the model depend on the coefficient of determination (R^2) and adjusted R^2 value. The smaller the difference between 1 and the R^2 value, the stronger the model (El Enshasy *et al.* 2008; Uppuluri *et al.* 2013b). The obtained values for R^2 (0.95714) and R adj (0.906) from the analysis of CCD experimental values show its accuracy and fitness. The strength of the model was exposed by response surface plots with respect to OTC yield and two other variables at a time (Uppuluri *et al.* 2013a,b). The correlation was plotted between observed and predicted values, showing values close to the diagonal line.

Table 4. Regression Analysis of the Central Composite Design

	Coefficient	p value	t value
Mean	45.09	0.000000	18.77
Maltose(L)	3.42	0.011560	3.02
Maltose(Q)	-4.06	0.098764	-1.80
Inoculum(L)	7.19	0.000048	6.44
Inoculum(Q)	-12.83	0.000074	-6.13
Caco3(L)	3.58	0.005087	3.48
Caco3(Q)	-2.51	0.028991	-2.50
Moisture(L)	2.78	0.026617	2.55
Moisture(Q)	-13.06	0.000008	-7.79
1L 2L	2.34	0.079716	1.93
1L3L	0.04	0.971803	0.03
1L4L	2.43	0.070230	2.00
2L3L	2.71	0.046919	2.23
2L4L	2.18	0.098571	1.80
3L4L	1.81	0.162993	1.49

Oxytetracycline production ranged from 5.01 to 0.45 mg /gds based on the experimental values. Critical values of the selected variables are given in Table 5.

Table 5. Critical Value Obtained through RSM (where the predicted OTC value is 5.02 mg/gds)

	Observed minimum	Critical values	Observed maximum
Maltose (g/10g)	0.08	1.25	1.60
Inoculum Size (ml/10g)	0.50	6.17	9.00
$CaCO_3$ (g/10g)	0.001	0.02	0.03
Moisture (%)	10.00	74.86	110.00

Real experiments were performed at critical values of selected variables and yield was found to be closer to predicted yield 5.02 mg /gds.

Antimicrobial Activity of Purified OTC

Purified OTC showed relatively good antimicrobial activity against various selected Gram positive and Gram negative organisms compared to the standard OTC (Table 6). The tested pathogens were sensitive to purified compound and further no growth was observed. This clearly demonstrated the bactericidal activity of purified OTC.

Table 6. Antimicrobial Spectrum (Zone of Inhibition) by Standard and Purified OTC

S.No	Reference strain	ZOI by Standard OTC	ZOI by Purified OTC
1	*Bacillus subtilis* MTCC 441	1.1 ± 0	1.0 ± 0
2	*Escherichia coli* MTCC 723	1.57 ± 0.0	1.22 ± 0.02
3	*Salmonella typhi* MTCC 531	2.6 ± 0	1.52 ± 0.02
4	*Staphylococcus aureus* MTCC 3160	1.87 ± 0.02	1 ± 0
5	*Shigella dysenteriae* ATCC 23513	2.37 ± 0.06	1.47 ± 0.02
6	*Klebsiella pneumoniae* MTCC 1667	2.4 ± 0	1.3 ± 0
7	*Pseudomonas aeruginosa* MTCC 2488	2.5 ± 0	1.67 ± 0.02

CONCLUSIONS

1. The present study exploited the use of *Prosopis juliflora* pods as a novel and cheap cellulosic substrate for the fermentative production of oxytetracycline (OTC).
2. High cellulosic content of these pods provides a very good platform for the growth of microorganisms, especially actinomycetes and fungi.
3. So, the cultivation of micro-organisms on these pods surely will be a value-added process capable of converting these materials into various biochemicals, especially biopharmaceuticals.
4. A universally accepted statistical optimization process (RSM), was used to further increase the OTC production from 0.4 to 5 mg/gds.
5. But still, scale-up needs to be done to develop a commercial process with techno-economical feasibility.

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