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# PRODUCTION AND OPTIMISATION OF TETRACYCLINE BY VARIOUS STRAINS OF *STREPTOMYCES* UNDER SOLID STATE FERMENTATION USING PINEAPPLE PEEL AS A NOVEL SUBSTRATE

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## Abstract

Pineapple peel is the principal solid waste product of the juice processing industry. The disposal of the fresh peels is becoming a major problem to many food processing industries. Dry pineapple peels are rich in biodegradable organic material and suspended solids; therefore, this waste was used as a novel substrate in present solid substrate fermentation. The effect of medium ingredients such as carbon, inorganic and organic nitrogen sources, inorganic salts on tetracycline production by various strains of *Streptomyces* [*S. aureofaciens* NCIM (2417, 2614, 2615), *S. rimosus* NCIM 2213 and *S. viridifaciens* NCIM 2506] in solid-state fermentations (SSF) was observed. The 1.0-fold lower antibiotic yield than the control in the presence of glucose and sucrose in SSF at 10% w/w was observed , whereas peanut meal and ammonium sulphate was found to be a more favorable organic and inorganic nitrogen source than the control in SSF at 10% and 1% w/w. Calcium carbonate ($CaCO_3$) as inorganic salt favored 0.5% higher antibiotic yield than the control at 1% w/w. Various crucial parameters such as initial moisture content, incubation temperature, initial pH, substrate particle size and inoculum size were derived; 65% moisture level, 35$^{o}$C temperature, pH 5-0-6.5, 6 x 4 mm particle size and (1.0 x $10^8$ spores/ ml) inoculum size was found to be suited for maximal tetracycline production. The maximum tetracycline production was observed following 3–7 days of fermentation cycle.

Keywords: Solid state fermentation, Tetracycline, Pineapple peel, Optimization

## Introduction

The tetracyclines are a family of polyketide antibiotics produced by *Streptomyces* genus of actinobacteria. The different form of tetracyclines are tetracycline, chlortetracycline demeclocycline, oxytetracycline and other most active types. These antibiotics are broad-spectrum in nature. At present, reports on the clinical use of tetracyclines have been generally confined to respiratory tract infection, sinuses, middle ear infection, urinary tract infections, intestinal infection and also gonorrhoea (*Chopra and Howe, 1978*, *Speer, et al 1992*). Moreover many derivatives of tetracycline such as doxycycline, lymecycline, meclocycline, methacycline, minocycline and rolitetracycline used in the treatment of chronic prostatitis, chlamydia and gum disease. More recently, tetracycline derivative (doxycycline) has been reported to be effective for the treatment for pelvic inflammatory disease (Gjonnaess et al, 1978). This makes tetracycline an attractive group of antibiotics with growing market.

Traditionally, tetracycline has been produced by submerged fermentation (SmF) and used in a one-way process in solution. In recent years, however Solid State Fermentation (SSF) processes have been increasingly utilized for the production of various antibiotics. SSF also holds tremendous potential for the production of antibiotics (Robinson et al, 2001). It can be of particular relevance in those processes where a crude fermented product may be used as an antibiotic source (Barrios-Gonzalez, et al 1988). The selection of a particular strain, however, remains a tedious task, particularly when commercially significant antibiotic yields are required. Agro-industrial residues are generally considered the best substrates for the SSF processes. The use of SSF for the production of antibiotic and other secondary metabolite has many advantages over submerged fermentation (Johns, 1992) and these have been widely discussed in the literature (Balakrshana and Panday,1996, Barrios-Gonzalez, et al 1993, Ohno, et al 1992)

Pineapple fruits are abundantly available in India. In 2009 the cultivation area of pineapple fruits was 80,000 hectares and annual production was 1, 17,832 tons. The pineapple peel wastes contain high concentration of biodegradable organic material and suspended solids. As a result it has a high BOD and withstanding at extreme pH conditions (Kroyer, 1991). The solid waste from pineapple canning process was estimated about 40 - 50 % from fresh fruit as pineapple peels and core (Buckle, 1989). With the goal of being economically competitive, pineapple peel a sugary

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agricultural waste was used in this article to produce tetracycline with various strains of *Streptomyces* by solid state fermentation. Inorganic salts, additional carbon source, inorganic and organic nitrogen source supplementation of the raw substrate in SSF may stimulate the growth or improve the process efficiency. There is a literature available on tetracycline production by SSF, where they were successfully produced tetracycline by using different solid substrate such as sweet potato residue, peanut shells, corn cob, corn pomace and cassava peels (Agenes, et al 2005, Yang and Swei, 1996, Yang and Yuan, 1990, Yang and Ling, 1989). From the above literature survey revel that absolutely no investigations available on use of pineapple peel wastes used as solid substrate for tetracycline production on SSF. The objective of the current study was the potential evaluation of various strains of Streptomyces for production of tetracycline using pineapple peel waste residues as substrate under solid-state fermentation. The culture conditions are initial moisture content, size of inoculum, initial pH, incubation period, substrate particle size; additional carbon source, inorganic and organic nitrogen source and inorganic salt were optimized for maximum tetracycline production by various spices of Streptomyces.

## Material and Methods

### Substrate and microorganism

Pineapple peel waste procured from a local food processing unit in Dharwad, India which was used as a solid substrate. It was dried at 60$^{o}$C for 72 h to reduce the moisture content to around 5%, and ground to the mean particle size (mm) of 12 x 4, 12 x 2, 6 x 4, 2.8–2.0; 2.0–1.4 and 1.4–1.0 were segregated and designated as A to F respectively. *Streptomyces* strains [*S. aureofaciens* NCIM (2417, 2614, 2615), *S. rimosus* NCIM 2213 and *S. viridifaciens* NCIM 2506] obtained from National Chemical Laboratory, Pune, India. These strains used throughout the study. *Bacillus cereus* NCIM 11778 was used in antimicrobial assay as test organism.

### Growth conditions

The culture was maintained on MGYP slants having the composition (%): malt extract 0.3, glucose 1.0, yeast extract 0.3, peptone 0.5 and agar 2.0. The pH of the medium was adjusted to 6.4 to 6.8 and culture was incubated at 30$^{o}$C for 48 h. Subculturing was carried out once in 2 weeks and the culture was stored at 4$^{o}$C.

### Inoculum preparation

A 5 ml of sterile water was added to the slant and the spores were scraped and transferred into 250 ml Erlenmeyer flask containing 50 ml of inoculum medium. The composition of the inoculum medium was; 2.5% soluble starch, 1.0% corn steep liquor, 0.5% $(NH_4)_2SO_4$, 2% $CaCO_3$, 1% NaCl, 1% $K_2HPO_4$, 1% $MgSO_4$ $7H_2O$ and pH 7.5. The flasks were incubated at 28$^{o}$C in shaker incubator (at 220 rpm) for 5 to 7 days. Spores were harvested and washed with sterile saline solution and resuspended in 25 ml sterile saline solution. This spore suspension was used as inoculum.

### Media preparation and fermentation

The basal solid medium contained pineapple peel waste 100 g and additional inorganic salts (1% w/w), inorganic nitrogen source (1% w/w), organic nitrogen source (10% w/w) and carbon source (10% w/w) were included. The contents were thoroughly mixed and autoclaved at 121$^{o}$C (15 psi) for 20 min. The sterilized medium is mixed thoroughly with spores of *Streptomyces* strains [*S. aureofaciens* NCIM (2417, 2614, 2615), *S. rimosus* NCIM 2213 and *S. viridifaciens* NCIM 2506] separately and incubated statically in flask (the thickness of medium was about 2 cm) at 35°C for 2-10 days by stirring once a day. All the experiments were performed in triplicate and mean values were reported as the % variation less than 5%.

### Extraction of antibiotics

The tetracycline from the fermented material was recovered by simple extraction method. For this, the fermented substrate was mixed thoroughly with 500 ml of distilled water and the contents are agitated for 1 h at room temperature in a rotary shaker at 150 rpm. At the end of extraction, the liquid was filtered off through Whatman No.1 filter paper and the resulting clear filtrate was used for tetracycline analysis (Agenes, et al 2005, Yang and Yuan, 1990).

### Analytical methods

The disc diffusion bioassay method that utilizes the antibacterial property of tetracycline to produce a zone of inhibition against *Bacillus cereus* NCIM 11778 as test organism (Grove and Randall, 1955, Pharmacopoeia of India, 2007) was used. The method employed the use of filter paper discs containing 10 µl of supernatant from fermentation broth of *Streptomyces* strains and negative control. These discs were dried and placed on the surface of agar plates seeded with *Bacillus cereus* NCIM 11778 strain. Positive control was consisted of disc with known amount of tetracycline. These plates were incubated at 37$^{o}$C for 24 h. Zones of inhibition were measured in mm. All experiments were conducted in triplicate, and the mean of the three is represented as milligram of tetracycline produced per gram of substrates. Standard tetracycline (Himedia, India) was used to construct the calibration curve. The moisture content of the pineapple peel waste was estimated by drying 100 g of pineapple peel waste to a constant weight at 105$^{o}$C

and the dry weight was recorded (Tran, et al 1998). To fix the initial moisture content of the solid medium, pineapple peel waste was soaked with the desired quantity of additional water. After soaking, the sample was again dried as described earlier and moisture percentage is calculated as follows, percent of moisture content (initial) of solid medium = (weight of the pineapple peel waste –dry wt.)×100 per dry wt. Initial pH of substrates is determined directly by immersing the pH electrode into the solid substrate (Yang and Swei, 1996).

### Effect of physical parameter on tetracycline

To study the effect of incubation period on tetracycline production, flasks were incubated for varying periods (3-10 days). Other parameters were kept at their optimum conditions (Ellaiah, et al 2004).

The effects of the inoculum on tetracycline production were studied by adding different concentration (($1x10^4$, $1x10^5$, $1x10^6$, $1x10^7$, $1x10^8$, $1x10^9$and $1x10^{10}$) of spores to the solid medium and fermentation was carried out for 7-8 days (Krishana and Nokes, 2001). The moisture content, pH of the substrate and incubation temperature was kept at their optimum levels.

To study the effect of incubation temperature on the production of tetracycline, the flasks were incubated at various temperatures (20, 25, 30, 35 and 40$^o$C). The other parameters like moisture content, pH of the substrate were kept at their optimum level and the fermentation was run for 7-8 days (Ellaiah, et al 2004).

Various moisture levels are employed in the substrate medium to study their effect on tetracycline production. Eleven flasks containing 100 g substrate were taken and distilled water was added to obtain various levels of moisture content (ranging from 30 to 80 %) (Sato, et al 1983). The fermentation was conducted as described in media preparation and fermentation.

The effect of initial pH of solid culture medium on production of tetracycline was studied by varying the pH of salt solution from pH 4-7. The pH was adjusted using 0.1 N hydrochloric acid or 0.1 N sodium hydroxide (Yang and Swei, 1996). The other conditions were moisture content of 65% and inoculums concentration of $10^8$ spores/ml and the fermentation was carried out for 7 days at 35°C.

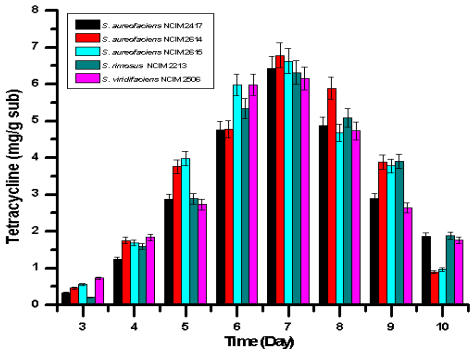
### Effect of additional nutrients on tetracycline production

The effects of various additional nutrients (carbon sources, inorganic and organic nitrogen sources and inorganic salt solution) on tetracycline production were studied by adding these to the pineapple peel waste substrate in separate experiments. Various carbon sources (soluble starch, sucrose, maltose, and glucose) and organic nitrogen source (peptone, soybean meal, peanut meal and beef extract) were added at a 10% (w/w) concentration to the solid substrate. Also various inorganic salts (sodium chloride, potassium dihydrogen phosphate, magnesium sulphate and calcium carbonate) and inorganic nitrogen source (ammonium nitrate. ammonium sulphate, ammonium chloride and potassium nitrate) were added at a 1% (w/w) concentration to the solid substrate (Agenes, et al 2005, Yang and Yuan, 1990).

## Results

Detectable tetracycline yield was attained on day 3 and maximum tetracycline secretion (6.43, 6.78, 6.63, 6.32 and 6.16 mg/g substrate) was achieved on day 7 of incubation by *Streptomyces* strains [*S. aureofaciens* NCIM (2417, 2614, 2615), *S. rimosus* NCIM 2213 and *S. viridifaciens* NCIM 2506], after day 7 there was decrease in tetracycline secretion (Fig. 1).

Fig. 1: Evaluation of fermentation period for the production of tetracycline from pineapple peel waste by using various strains of *Streptomyces* in SSF




Maximum tetracycline secreted (12.9, 12.78, 12.7, 12.94 and 12.45 mg/g substrate) at $10^8$ spores /ml by *Streptomyces* strains [*S. aureofaciens* NCIM (2417, 2614, 2615), *S. rimosus* NCIM 2213 and *S. viridifaciens* NCIM 2506] (Fig. 2) on day 7 of incubation. A higher or lower than $10^8$ spores /ml will not favor the tetracycline secretion.

Fig. 2: Evaluation of inoculums size for the production of tetracycline from pineapple peel waste by using various strains of *Streptomyces* in SSF

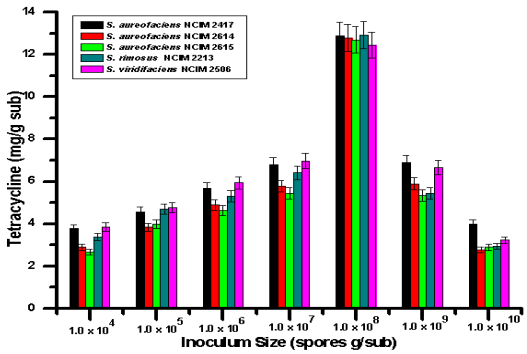
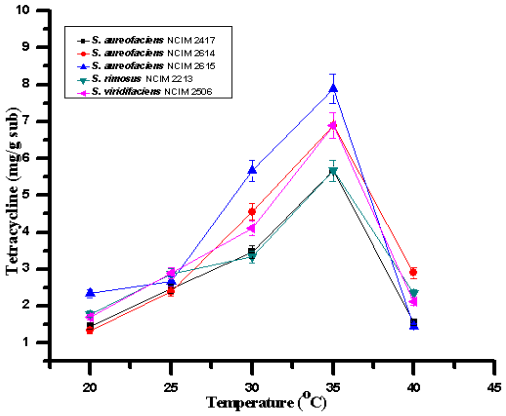


Fig. 3: Evaluation of incubation temperature for the production of tetracycline from pineapple peel waste by using various strains of *Streptomyces* in SSF




The influence of incubation temperature on tetracycline secretion was studied by monitoring in the temperature range between 20 to 40°C. Maximum tetracycline secretion (5.67, 6.89, 7.89, 5.67 and 6.89 mg/g substrate) at 35°C with *Streptomyces* strains [*S. aureofaciens* NCIM (2417, 2614, 2615), *S. rimosus* NCIM 2213 and *S. viridifaciens* NCIM 2506] (Fig. 3). Reduced tetracycline secretion was observed when incubation temperature is higher or lower than 35°C.

The maximum tetracycline secretion (6.67, 5.67, 5.90, 5.92 and 6.90 mg/g substrate) was achieved at 65% initial moisture content by *Streptomyces* strains [*S. aureofaciens* NCIM (2417, 2614, 2615), *S. rimosus* NCIM 2213 and *S. viridifaciens* NCIM 2506] on day 7 of incubation ( Fig 4). A decrease in tetracycline secretion was observed, when the moisture contents was higher or lower than 65% initial moisture content.

Fig. 4: Evaluation of initial moisture for the production of tetracycline from pineapple peel waste by using various strains of *Streptomyces* in SSF

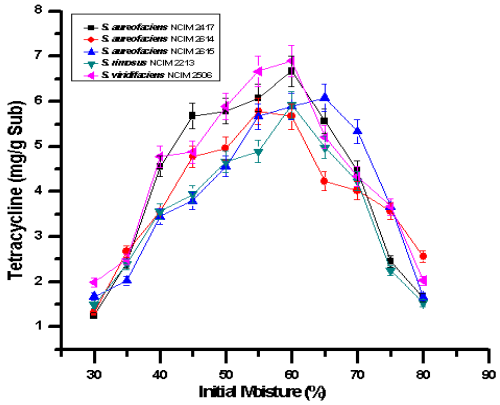



Fig. 5: Evaluation of various initial pH for the production of tetracycline from pineapple peel waste by using various strains of *Streptomyces* in SSF

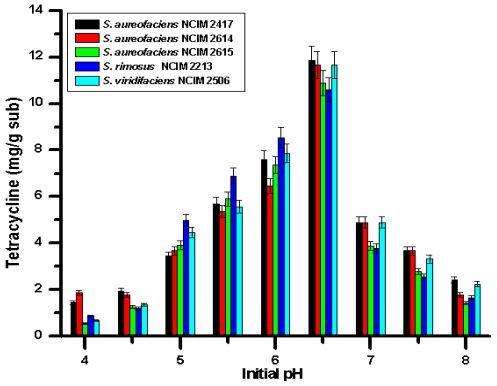
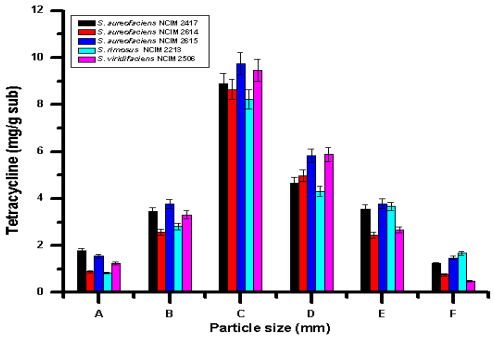



Fig. 6: Evaluation of substrate particle size for the production of tetracycline from pineapple peel waste by using various strains of *Streptomyces* in SSF




The effect of initial pH of the substrate on tetracycline secretion was shown in Fig. 5. Evaluation of data at different initial pH conditions indicated that pH 6 to 6.5 is suitable for tetracycline production and maximum tetracycline secretion (11.85, 11.67, 10.89, 10.6 and 11.67 mg/g substrate) with *Streptomyces* strains [*S. aureofaciens* NCIM (2417, 2614, 2615), *S. rimosus* NCIM 2213 and *S. viridifaciens* NCIM 2506] was noticed at pH 6.5. Higher or lower initial pH resulted in reduced antibiotic secretion than initial pH 6.5.

The effect of particle size of the substrate on tetracycline secretion was shown in Fig. 5. The selected particles sizes "C" (6 x 4 mm) supported maximum tetracycline (8.9, 8.65, 9.75, 8.23 and 9.46 mg/g substrate) production with *Streptomyces* strains [*S. aureofaciens* NCIM (2417, 2614, 2615), *S. rimosus* NCIM 2213 and *S. viridifaciens* NCIM 2506]. A 30% improvement was observed in tetracycline production on optimization of particle size.

Fig. 7: Effect of inorganic salts (1% w/w) for the production of tetracycline from pineapple peel waste by using various strains of *Streptomyces* in SSF

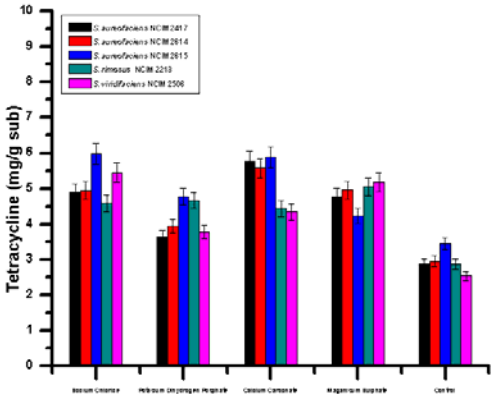



Fig. 8: Effect of inorganic nitrogen source (1% w/w) for the production of tetracycline from pineapple peel waste by using various strains of *Streptomyces* in SSF

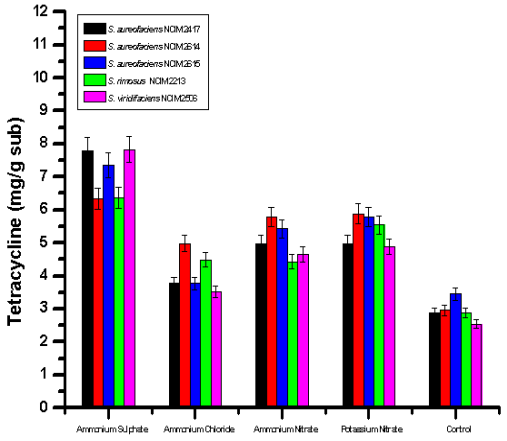



Fig. 9: Effect of organic nitrogen source (10% w/w) for the production of tetracycline from pineapple peel waste by using various strains of *Streptomyces* in SSF

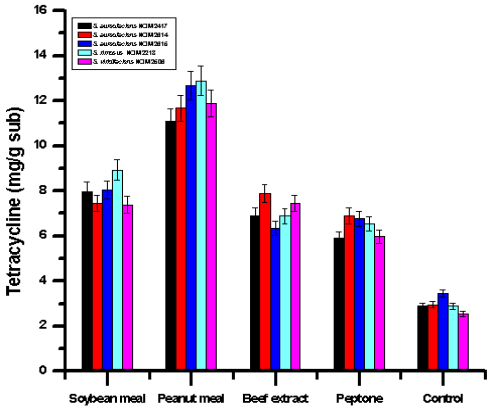
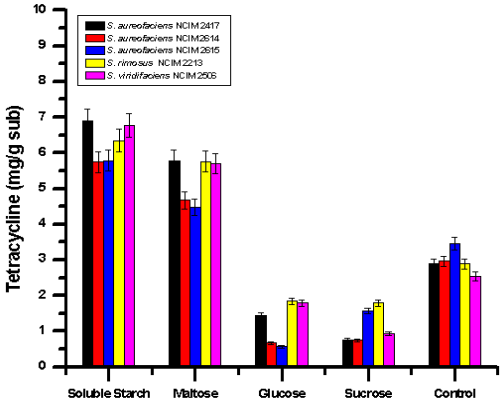



Fig. 10: Effect of additional carbon source (10% w/w) for the production of tetracycline from pineapple peel waste by using various strains of *Streptomyces* in SSF




The effect of inorganic salts supplementation on tetracycline production was shown in Fig. 7. Addition of 1 % (w/w) $CaCO_3$ resulted in the maximal tetracycline secretion (5.78, 5.58, 5.89,4.44 and 4.34 mg/ g substrate) with *Streptomyces* strains [*S. aureofaciens* NCIM (2417, 2614, 2615), *S. rimosus* NCIM 2213 and *S. viridifaciens* NCIM 2506] than control. The presence of 1% (w/w) NaCl and $MgSO_4$ moderately enhanced the tetracycline secretion.

The effect of different inorganic nitrogen sources 1% (w/w) on tetracycline production was investigated using pineapple peel waste as solid substrate. Amongst inorganic nitrogen sources used ammonium sulphate supported maximum tetracycline production (457.8, 6.34, 7.36, 6.38 and 7.83 mg/g substrate)) with *Streptomyces* strains [*S. aureofaciens* NCIM (2417, 2614, 2615), *S. rimosus* NCIM 2213 and *S. viridifaciens* NCIM 2506 than control. Other organic nitrogen source 1% (w/w) such as ammonium nitrate and potassium nitrate moderately increases the tetracycline production compared to control.

The impact of supplementation of various organic nitrogen sources at 10% (w/w) concentration on tetracycline production was studied and the results are shown in Fig. 9. All the supplemented organic nitrogen sources were found to enhance the tetracycline secretion. Among all organic nitrogen sources peanut meal enhances maximum antibiotic secretion (11.1, 11.67,12.67,12.89 and 11.87 mg/g substrate) than control

The maximum tetracycline secretion (6.89, 5.74, 5.78, 6.34 and 6.77 mg/g substrate) was achieved by *Streptomyces* strains [*S. aureofaciens* NCIM (2417, 2614, 2615), *S. rimosus* NCIM 2213 and *S. viridifaciens* NCIM 2506], when 10% (w/w) soluble starch was used as additional carbon source in the fermentation medium. A 10% (w/w) of glucose and sucrose inhibited tetracycline secretion than control.

## Discussion

Antibiotics are traditionally produced by submerged fermentation, and their yields tend to be low due to the energy input (Tomasini, et al 1997). The advantages of solid-state fermentation include (i) it is more competitive process, and it may be a viable option for the industrial production of secondary metabolites (Robinson, et al 2001), (ii) It requires lower manufacturing cost by utilizing unprocessed and moderately processed raw materials (Adinarayana, et al 2003), (iii) it is less sensitive to contamination when compared to submerged fermentation (Grohmann, 1993). SSF has successfully achieved higher titers of antibiotics (Farzana, et al 2005, Robinson, et al 2001), so this technique has encouraged us for tetracycline production. Moreover, submerged fermentation is usually employed for commercial production of tetracycline (Mamoru, et al 1986). The mechanism of tetracycline secretion in solid-state fermentation is probably the same as that proposed in submerged fermentation.

Provision of optimum level of inoculum is also very critical in SSF. Lower and higher inocula levels than the optimum level, resulted in decreased tetracycline activities (Fig. 2). Available reports on the role of inoculum size on tetracycline secretion is not so similar (Yang and Yuan, 1990). A low inoculum density may give insufficient biomass causing reduced product formation, where as higher inoculum than optimum may produce too much biomass and may deplete the nutrients necessary for secondary metabolite production (Mudgetti, et al 1992). From the results obtained it was evident that maximum tetracycline production was obtained at 35°C, which clearly indicated the mesophilic nature of the *Streptomyces* strains (Fig 3). In previous reports (Agenes, et al 2005, Yang and Swei, 1996, Yang and Yuan, 1990, Yang and Ling, 1989) says that optimal temperature range between 26 to 35°C for tetracycline secretion by *Streptomyces* strains in SSF.

The highest tetracycline production was obtained at 65% initial moisture contents (Fig.4) Low moisture levels decreases the solubility and availability of nutrients, minimize heat exchange and oxygen transfer rates thus lowering the activity of microbial cultures and resulting in reduced productivity (Carrizales, et al 1981, Pandey, et al 2001, Pandey, et al 2000, Pandey, 1994, Pandey, 1992). Higher substrate moisture in SSF resulted in less productivity because of reduced mass transfer process such as diffusion of solutes and gases to the cells during fermentation process (Carrizales, et al 1981, Pandey, et al 2000, Pandey, 1994).The *Streptomyces* strains could adapt initial pH range from 5 to 7 and optimum level at pH 6.5. As the metabolic activities of the *Streptomyces* are very much sensitive to the initial pH change (Agenes, et al 2005, Yang and Swei, 1996, Yang and Yuan, 1990, Yang and Ling, 1989). Evaluation of the optimum levels of initial pH is very important for overall economic feasibility of the production process. The particles sizes "C" (6 x 4 mm) of substrate was found to be optimal size of the substrate for maximum tetracycline production (Fig 6). Variation of substrate particle size resulted in reduction of antibiotic production. The particle size of substrate used in present investigation is in quite similar with previous literature reported with other microbial strains in SSF. (Tran, et al 1998, Venkateshwarlu, et al 2000, Yang and Yuan, 1990).

However, previously it has been reported (Agenes, et al 2005, Yang and Swei, 1996, Yang and Yuan, 1990, Yang and Ling, 1989) that maximum tetracycline production was shown in the presence of 1% (w/w) $CaCO_3$. In the present experiments, apparently 1% (w/w) $CaCO_3$ enhances maximum tetracycline production compared to control. Tetracycline production by different *Streptomyces* strains in SSF was supported by the presence of various inorganic nitrogen sources (1% w/w) such as ammonium chloride, ammonium sulphate, ammonium nitrate (Agenes, et al 2005, Yang and Swei, 1996, Yang and Yuan, 1990, Yang and Ling, 1989). Ammonium sulphate (1% w/w) enhances maximum tetracycline production as compared to control (Fig. 8), even ammonium nitrate and potassium nitrate also shown significant effect on tetracycline production. All the supplemented organic nitrogen sources were found to enhance the tetracycline production. Peanut meal (Fig 9) and other organic nitrogen source 10% (w/w) such as beef extract and soybean meal significantly increases the tetracycline production as compared to control. Available reports on the role of organic nitrogen sources on tetracycline secretion was found to be similar to our data (Agenes, et al 2005, Yang and Swei, 1996, Yang and Yuan, 1990, Yang and Ling, 1989). It has been reported that additional carbon source slightly stimulated tetracycline production (Agenes, et al 2005, Yang and Yuan, 1990). Moreover, in case of other antibiotics yield was increased by the addition of different carbon sources as additives in fermentation medium was reported (Adinarayana, et al 2003, Mahalaxmi, et al 2010, Sekar, et al 1997). Soluble starch (10% w/w) was used as additional carbon source in SSF was moderately increased, but addition of external carbon sources did not improve tetracycline yield in SSF by various *Streptomyces* strains (Fig. 10).

## Conclusion

Pineapple peel waste was found to be the best novel solid substrate for the production of tetracycline by the *Streptomyces* strains in SSF. The initial moisture content, initial pH, inoculum size, substrate particle size, incubation period and incubation

temperature factors were found have significant effect on the growth of *Streptomyces* strains and tetracycline production. Supplementation of pineapple peels waste with additional inorganic salts; organic and inorganic nitrogen sources proved to be beneficial and increased the yield of tetracycline after optimization of the nutritional parameters. But carbon source doses not shows significant effect on tetracycline production. Therefore, the Pineapple peel waste could be successfully used as novel solid substrate to produce tetracycline antibiotic under optimized SSF parameters to achieve a very good yield.

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