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**APPLE POMICE AND ORANGE PEEL AS SUPPORT FOR SOLID STATE  
FERMENTATION TO PRODUCE TETRACYCLINE FROM *STREPTOMYCES  
REMOSUS* NCIM 2213**

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### Abstract

Tetracycline production by a *Streptomyces remosus* NCIM 2213 was optimized using apple pomice and orange peel in solid state fermentation (SSF). Different fermentation parameters such as incubation temperature, initial moisture content, initial pH, particle size, incubation time, inoculum, additional carbon, organic and inorganic nitrogen source were investigated. Both apple pomice and orange peel supported good *Streptomyces remosus* NCIM 2213 growth and antibiotic production as evident by its chemical composition. Maximum tetracycline production was observed at 60% substrate moisture, a incubation of day 8, inoculums size  $4 \times 10^8$  spore/ml, incubation temperature 30°C, particle size 0.84 mm and a initial substrate pH of 6.0, respectively. Antibiotic production was stimulated by supplementing the apple pomice and orange peel with 20% w/v each of maltose as carbon source, malt extract as organic nitrogen source and inorganic nitrogen source as NaNO<sub>2</sub>.

**Keywords:** Tetracycline, *Streptomyces remosus* NCIM 2213, Solid state fermentation, Apple pomice, Orange peel, Optimization.

### Introduction

The technique of solid-state fermentation (SSF) involves the growth and metabolism of microorganisms on moist solids in the absence or near absence of any free-flowing water. This technique offers distinct advantages

over submerged fermentation (SmF) including economy of the space needed for fermentation; simplicity of the fermentation media; no requirement for complex machinery, equipments and control systems; greater compactness of the fermentation vessel owing to a lower water volume; greater product yields; reduced energy demand; lower capital and recurring expenditures in industry; easier scaleup of processes; lesser volume of solvent needed for product recovery; superior yields; absence of foam build-up; and easier control of contamination due to the low moisture level in the system. Additionally, such a system finds greater application in the areas of solid waste management, biomass energy conservation, and in the production of secondary metabolites; however, SSF also has some limitations such as a limited choice of microorganisms capable of growth at a reduced moisture level, exacting demand for monitoring and control of parameters such as temperature, pH, humidity, air flow, free oxygen transfer, and removal of CO<sub>2</sub> generated during fermentation, and the need for pretreatment of certain solid substrates<sup>1, 2,3, 4, 5</sup>. The nature of the solid substrate also plays an important role in antibiotic productivity. The screening of several agro-industrial residues is usually needed to select the most appropriate substrate. Their cost and availability are also factors to be considered when choosing a substrate.

Apple is grown worldwide including India (FAO) and around 30 % of the total yield is converted into byproduct of juice extraction industries and being highly biodegradable; its disposal is a serious problem. Apple pomace (AP) is a heterogeneous mixture consisting of peel, core, seed, calyx, stem and soft tissues. Since it is a rich source of carbohydrates, acids, fibers, vitamin C and minerals, its disposal as a waste in the environment is a huge loss of precious natural resources<sup>6,7</sup>. However, its nitrogen deficient nature<sup>6,7</sup> makes it inadequate as an animal feed. Orange peels (OP) are the main solid by-product of the citrus processing industry and constitute about 50% of fresh fruit weight. The disposal of the fresh peels is becoming a major problem in many fruit processing industries. Usually, fruit processing industries dry the residue and give it away as raw material for pectin extraction or is pelletized for animal feeding, though none of these processes is profitable. As for other food processing wastes, various microbial transformations have been proposed for citrus peels, producing valuable products like biogas, ethanol, citric acid, chemicals, various enzymes, volatile flavouring compounds, fatty acids and microbial biomass<sup>8</sup>. Therefore, its utilization in one or other forms is the immediate necessity from the economic and

environmental protection point of view, hence it can serve as an important natural substrate for tetracycline production.

Tetracyclines are important derivatives of polycyclic naphthacene carboxamide antibiotics used widely in pharmaceutical preparations. These antibiotics were discovered in the 1940s and exhibited activity against a wide range of microorganisms including gram-positive and gram-negative bacteria, chlamydiae, mycoplasmas, rickettsiae, and protozoan parasites. They are inexpensive antibiotics, which have been used extensively in the prophylaxis and therapy of human and animal infections and also at subtherapeutic levels in animal feed as growth promoters<sup>9</sup>.

Actinomycetes systems are being increasingly investigated for the production of antibiotics and other metabolites by SSF. These fermentation systems, which are closer to the natural habitats of microbes, may prove more efficient in producing certain metabolites. Actinomycetes belonging to the genus *streptomyces* have been used throughout the world for production of tetracyclines. Reports on actinomycetes antibiotics by SSF, however, are primarily confined to only *streptomyces* spp which could be attributed to their ability to adhere to the substrate particles to produce filamentous cells for penetration, and to their specific need for water activity. Among these, *Streptomyces remosus* NCIM 2213 antibiotic are reported to have higher productivity. There are very few reports on tetracycline production in SSF to date. The aim of this study was to production and optimization of tetracycline from apple pomace and orange peels in solid-state fermentation using a culture of *Streptomyces remosus* NCIM 2213.

## **Material and methods**

### **Microorganism**

A new strain of *Streptomyces remosus* NCIM 2213 isolated procured from national chemical laboratory Pune, India, was used in the present study. It was maintained on MGYE (maltose, glucose, yeast extract and peptone) agar slants at 4 °C. Test organism *Staphylococcus aureus* NCIM 2901 isolated procured from national chemical laboratory Pune, India, was used in the present study.

### **Substrate procurement and pre-treatment**

Two different agricultural waste substrates, such as AP and OP were chosen as substrates for SSF in the present study. AP and OP were procured from the local fruit processing industry, Dharwad, Karnataka, India, dried, milled and passed through a sieve shaker to obtain the material of various particle sizes of 0.21, 0.42, 0.63, 0.84 and 1.5mm. Substrate with particle size range of about 0.21mm to 1.5mm was used for production of tetracycline. All the chemicals used in the present study were of analytical grade and procured from Fisher Scientific (Mumbai, India), Sigma–Aldrich (St. Louis, MO, USA) and Hi media (Mumbai, India).

### **Inoculum preparation**

*Streptomyces remosus* NCIM 2213 was grown on MGYB medium for 5 days (30°C). The spores produced were harvested with 0.05% Tween 80 in water and the suspension obtained was used to inoculate the culture medium for SSF studies.

### **Optimization of fermentation process under SSF**

Various process parameters influencing antibiotic production during SSF were optimized. The strategy followed was to optimize each parameter, independent of the others and subsequently optimal conditions were employed in all experiments. In a sequential order, the various process parameters were optimized for maximal antibiotic production as follows: incubation time (2, 4, 6, 8, and 10th day), initial moisture content (30, 40, 50, 60 and 70%), initial pH (4.5, 5.0, 5.5, 6.0 and 6.5), inoculum concentration (10, 15, 20, 25 and 30%), incubation temperature (20, 25, 30, 35 and, 40 °c) and supplementation of additional carbon source (glucose, sucrose, maltose, lactose, starch, mannitol, sorbitol at 20% w/v), additional organic nitrogen (peptone, yeast extract, malt extract, beef extract at 20% w/v) and inorganic nitrogen sources (ammonium sulphate, ammonium chloride, sodium nitrate, potassium nitrate at 20% w/v).

### **Solid-state fermentation**

Ten grams of the dried substrate taken in a cotton plugged 250 ml Erlenmeyer flask were supplemented with 3.0 ml of salt solution containing (%)  $\text{NH}_4\text{NO}_3$  0.5,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.1 and NaCl 0.1. The substrate moisture was adjusted to the required level by adding distilled water. Substrates were sterilized at 121 °C and 15 psi for 15 min,

cooled and inoculated with 1.0 ml spore suspension ( $5 \times 10^6$  spores per ml) of *Streptomyces reimosus* NCIM 2213 strain. The flasks were incubated at 30 °C for 96 h unless otherwise mentioned. All experiments were carried out in three sets.

#### **Assay of moisture content**

Five grams of solid medium or fermented product was dried to constant weight at 105 °C, and the dry weight was recorded. The moisture content was calculated as follows:

Moisture content of solid medium or fermented product (%) = ((weight of solid medium or fermented product - dry weight)/( dry weight)) X100

#### **Determination of pH value**

Ten milliliters of distilled water were added into 1.0 g of medium, and then it was agitated violently. After 15 min, the pH of the supernatant was measured with a pH meter.

#### **Antibiotic extraction**

After incubation, the culture was extracted with 30 ml distilled water at  $28 \pm 2$  °C on an orbital shaker at 180 rpm for 2 h. The extract was centrifuged at 10,000g for 10 min and ml of supernatant was extracted with 4 ml of methanol to precipitate the proteins. The precipitated proteins were removed by centrifugation at 10,000g for 10 min and the supernatant was filtered through 0.45 µm membrane filters (Millex\_-HV, Millipore, USA).

#### **Turbidimetric antibiotic assay**

Turbidimetric antibiotic assay is based on the change or inhibition of growth of a test microorganisms in a liquid medium containing a uniform concentration of an antibiotic. After incubation of the test organism (*Staphylococcus aureus* NCIM 2901) in the working dilutions of the antibiotics, the amount of growth is determined by measuring the light transmittance using spectrophotometer. The concentration of antibiotic is determined by comparing amounts of growth obtained with that given by the reference standard antibiotic solutions. Use of this method is appropriate only when test samples are clear<sup>10,11</sup>.

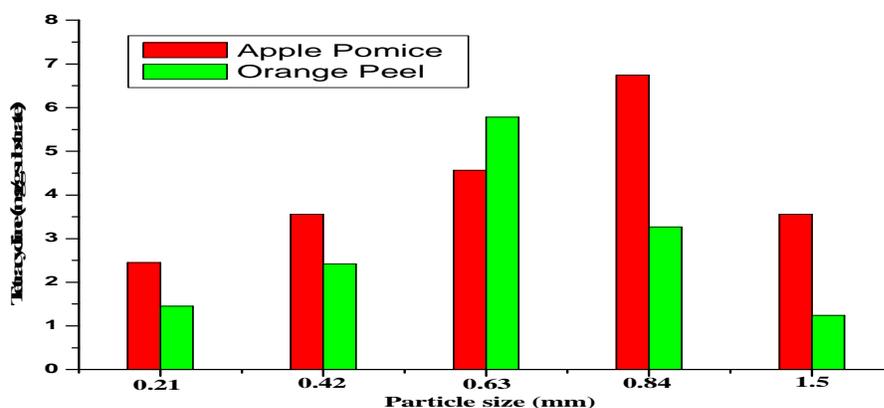
## Results and discussion

### Selection of the substrate

In SSF, the selection of a suitable solid substrate for a fermentation process is a critical factor and thus involves the screening of a number of agro-industrial materials for microbial growth and product formation. In the present studies, two substrates, viz. apple pomace and orange peel were used to prepare for growth and tetracycline production by the *Streptomyces remosus* NCIM 2213 in 2 week. All the substrates supported growth and antibiotic formation by *Streptomyces remosus* NCIM 2213, although apple pomace media proved superior to orange peel.

### Effect of particle size

Among the selected particles sizes designated as 0.84 mm in case of apple pomice (6.74 mg/g sub) and 5.78mm in case of orange peel (5.78 mg/g sub) supported maximum tetracycline. A 30% (4.56-6.74 mg/g sub) in case of apple pomice and 20% (2.42-5.78 mg/g sub) in case orange peel improvement was observed in tetracycline production on optimization of particle size. Fine particle size (<0.42 mm) yielded very little tetracycline. This indicated that the intermediate and coarse particles provided good support for the organism, which in turn gave high tetracycline yields. Variation from this substrate particle size resulted in reduction of antibiotic production (Fig. 1). This data is in accordance with literature reported particle size mediated microbial product production under SSF with different microbial strains<sup>12, 13, 14</sup>. Such data suggest that selection of proper size of particles is one of the essential requirements in SSF for optimum production as noticed by Prakasham *et al.*, 2006<sup>12</sup>.



**Fig. 1.** Effect of particle size (mm) on tetracycline production by *Streptomyces remosus* NCIM 2213 using apple pomice and orange peel under solid state fermentation.

### Effect of incubation initial moisture content

The study for initial moisture content suggested the experiment with 60% initial moisture content was the best for tetracycline production with tetracycline yield of (5.78 mg/g sub) in case of apple pomice and(4.68 mg/g sub) in case of orange peel (Fig. 2). The moisture content has an important role in solid state fermentation and although fermentation with relatively no moisture to very high initial moisture levels are reported<sup>15</sup> however it has been observed that high moisture content leads to aggregation of substrate particles, poor aeration, and possible anaerobic conditions<sup>16</sup> while very low moisture content restricts the fungal growth<sup>17</sup>. Optimal moisture content depends on the nature of microorganism and the substrate being used. For tetracycline production 50-70% initial moisture produced maximum yield by *Streptomyces remosus* NRRL B1679<sup>18</sup>.

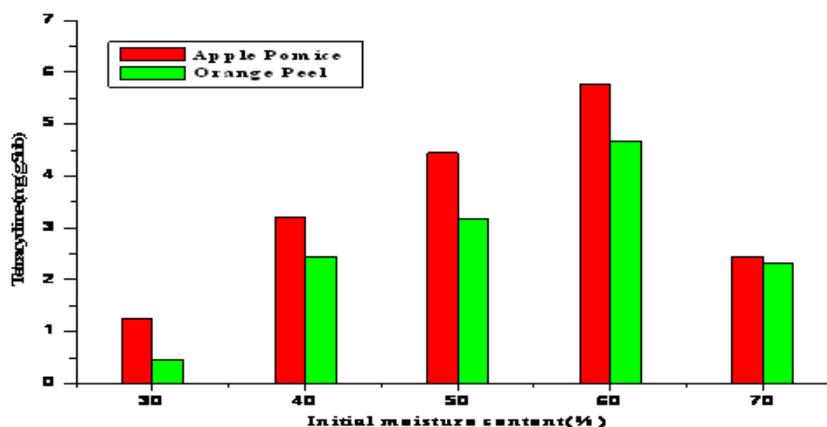
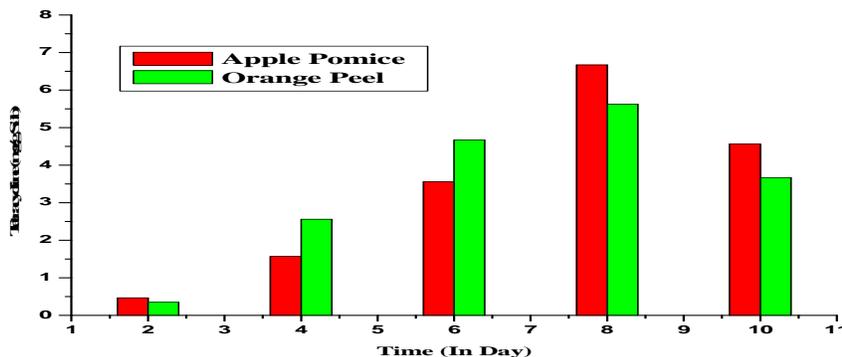


Fig. 2. Effect of initial moisture content (%) on tetracycline production by *Streptomyces remosus* NCIM 2213 using apple pomice and orange peel under solid state fermentation.

### Effect of incubation time

After inoculation, the flasks were incubated at 30 °C and the tetracycline production was measured after different time periods. The tetracycline production started after day 2 [(0.46mg/g sub) in case of apple pomice and (0.35 mg/g sub) in case of orange peel] incubation and increased with time peaking at day 10 [(6.67 mg/g sub) in case of apple pomice and (5.63 mg/g sub) in case of orange peel]. Thereafter, the tetracycline production started decreasing (Fig. 3). Maximum yield of tetracycline was obtained after day 8 of incubation at 30 °C. Decrease in production after day 8 may be due to accumulation of end product which hampers tetracycline production or may

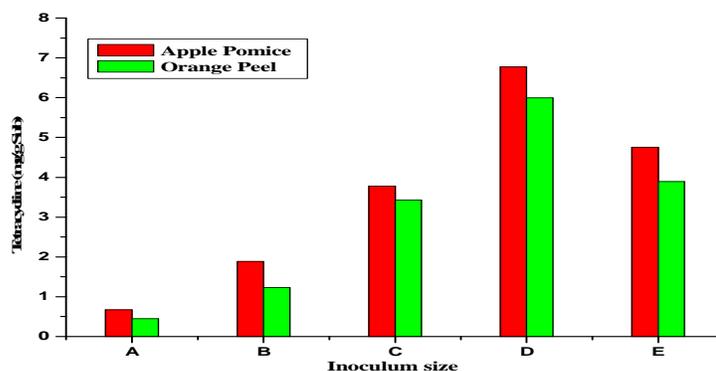
be due to accumulation of toxic metabolites secreted during fermentation (19). Agnes et al., (2005)<sup>18</sup> reported maximum production after day 5 of incubation with *Streptomyces sp. OXC1* under solid state fermentation.



**Fig. 3.** Effect of incubation time ( In days) on tetracycline production by *Streptomyces remosus* NCIM 2213 using apple pomice and orange peel under solid state fermentation.

### Effect of inoculums

The inoculum level was also an important factor for the production of tetracycline. High inoculum levels are inhibitory in nature. The highest tetracycline production (6.78 mg/g sub in case of apple pomice and 5.99 mg/g sub in case of orange peel) was obtained at an inoculum level of  $4 \times 10^8$  spore/ml. Maximum production of tetracycline was obtained with  $4 \times 10^8$  spore/ml showing maximum production of ( 6.78 mg/g sub in case of apple pomice and 5.99 mg/g sub in case of orange peel) tetracycline. A decrease in metabolite production was noted when the inoculum size decreased (Fig. 4). Antibiotic production attains its peak when the nutrients available to the biomass are balanced. When there is an imbalance between nutrients and proliferating biomass, the metabolic synthesis decreases, which is true with all microorganisms<sup>20</sup>.



**Fig. 4.** Effect of inoculum level (A= $1 \times 10^8$ , B= $2 \times 10^8$ , C= $3 \times 10^8$ , D= $4 \times 10^8$ , and E= $5 \times 10^8$  Spore/ml) on tetracycline production by *Streptomyces remosus* NCIM 2213 using apple pomice and orange peel under solid state fermentation.

## Effect of incubation temperature

Incubation temperature was shown to effect tetracycline production. It was another critical parameter that has to be controlled which varies from organism to organism<sup>3</sup>. Optimal temperature for maximum tetracycline production (5.78 mg/g sub incase of apple pomice and 5.34 mg/g sub in case of orange peel) was found to be 30 °C. The results are shown in Fig. 5.

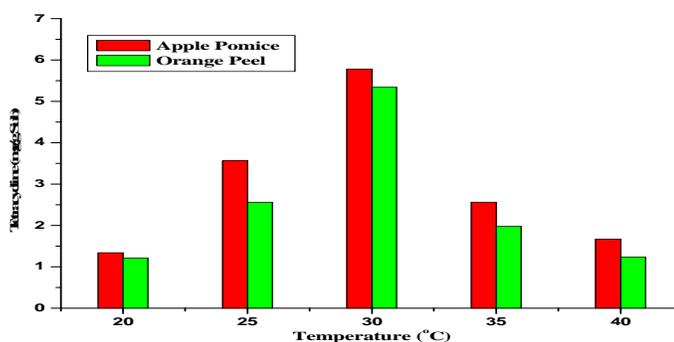


Fig. 5. Effect of incubation temperature on tetracycline production by *Streptomyces remosus* NCIM 2213 using apple pomice and orange peel under solid state fermentation.

## Effect of Initial pH

The pH of the medium strongly affects many enzymic processes and transport of various components across the cell membrane<sup>21</sup>. In order to study the effect of pH of the medium on antibiotic production in SSF, experiments were performed with media of different initial pH and incubated for 10 days. Antibiotic production gradually increased with increase in the pH of the medium from 5.0 and maximum production (6.79 mg/g sub in casa of apple pomice and 6.34 mg/g sub in case of orange peel) was recorded at pH 6.0 (Fig. 6). The antibiotic yield decreased if the medium pH was higher than this. Similar observations were made by Shang-Shyng Yang and Meei-Yueh Ling, (1989)<sup>22</sup> for tetracycline production in Sweet Potato Residue -based SSF medium.

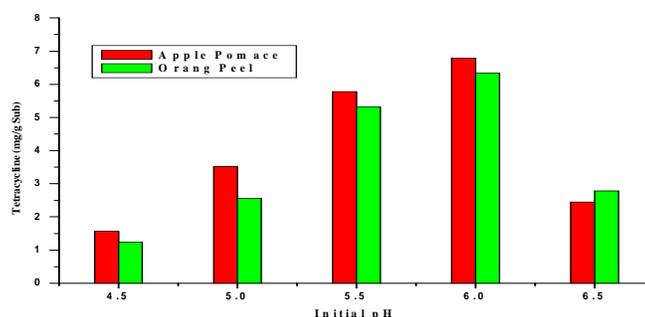


Fig. 6. Effect of initial pH on tetracycline production by *Streptomyces remosus* NCIM 2213 using apple pomice and orange peel under solid state fermentation.

### Effect of additional carbon source

Carbon source represents the energy source that will be available for the growth of the microorganism. The influence of additional carbon sources (20% w/v) was studied. Of the carbon sources tested, soluble starch increased the tetracycline production (6.78 mg/g sub incase of apple pomice and 6.34 mg/g sub incase of orange peel) followed by maltose, lactose, mannitol, sorbitol glucose and sucrose compared to control (Fig. 7). Earlier workers reported soluble starch as the best carbon supplement for tetracycline production in *Streptomyces sp. OXC1*<sup>18</sup> and *Streptomyces viridifaciens* ATCC 11989<sup>22</sup>.

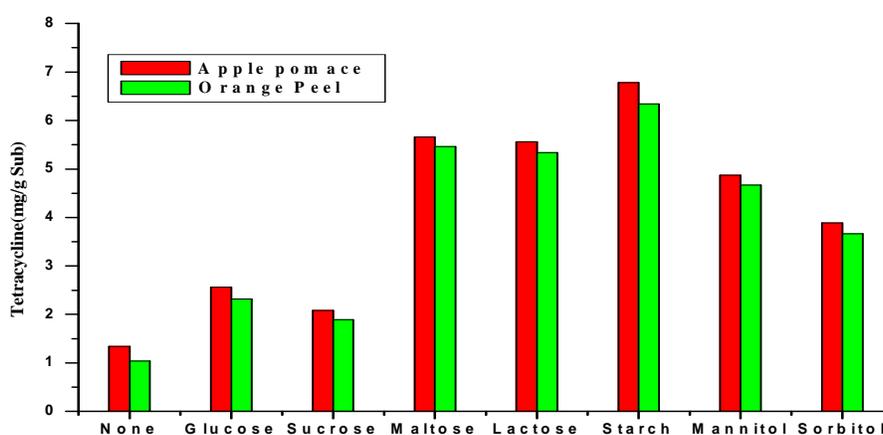


Fig. 7. Effect of additional carbon source (20% w/v) on tetracycline production by *Streptomyces remosus* NCIM 2213 using apple pomice and orange peel under solid state fermentation.

### Effect of additional organic nitrogen source

Organic nitrogen can be an important limiting factor in the microbial production of antibiotics<sup>13</sup>. The effect of supplementation with various organic nitrogen sources on tetracycline production by *Streptomyces remosus* NCIM 2213 is shown in (Fig. 8). Organic nitrogen sources in the basal medium were at a level of 20% (w/v). All organic nitrogen sources showed a general increase in the tetracycline production as compared to control. It was seen that peptone, yeast extract and beef extract stimulated the production of tetracycline. The highest concentration of tetracycline of 6.37 mg/g sub incase of apple pomice and 6.21 mg/g sub incase of orange peel was observed with malt extract.

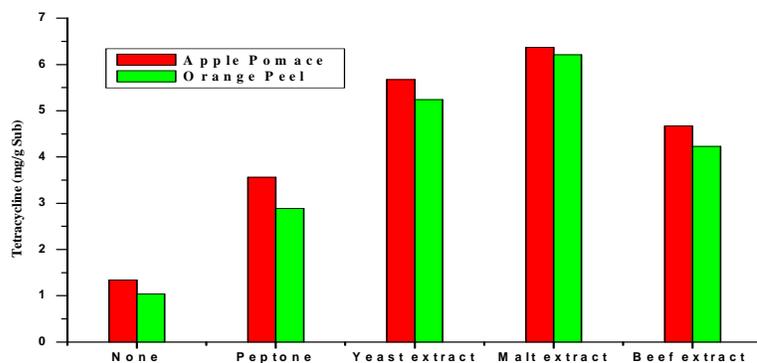


Fig. 8. Effect of additional organic nitrogen source (20% w/v) on tetracycline production by *Streptomyces remosus* NCIM 2213 using apple pomace and orange peel under solid state fermentation.

### Effect of additional inorganic nitrogen source

Type and nature of inorganic nitrogen sources are among the most important factors for any fermentation process<sup>23</sup>. Fig. 9 shows the effect of supplementation of the substrate with inorganic nitrogen source on tetracycline production. All the compounds exerted a beneficial impact on tetracycline synthesis by the *Streptomyces remosus* NCIM 2213 culture. However, among the compounds supplemented, one of the inorganic nitrogen sources, sodium nitrate, resulted in 6.48 mg/g sub incase of apple pomace and 6.06 mg/g sub incase of orange peel, which was better than the control of substrate without addition of inorganic nitrogen sources source.

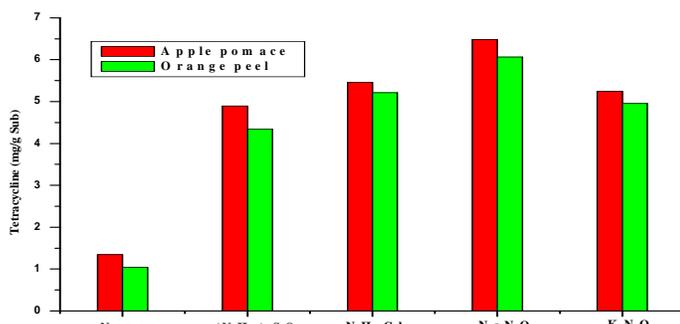


Fig. 9. Effect of additional inorganic nitrogen source (20% w/v) on tetracycline production by *Streptomyces remosus* NCIM 2213 using apple pomace and orange peel under solid state fermentation.

### Conclusion

The results described in this paper, for the first time, showed that *Streptomyces remosus* NCIM 2213 could be cultivated under SSF for the tetracycline using as apple pomace and orange peel as major solid substrate. Some medium components and fermentation parameters were found to play very significant roles in enhancing the growth of microbes and tetracycline. The optimal solid medium was determined to be (20% w/v) maltose, malt extract and

sodium nitrate. . Higher tetracycline production was obtained by using a selected physical condition of substrate initial moisture (60%), particle size (0.84mm), inoculum level (  $4 \times 10^8$  spore/ ml), initial pH (6.0), incubation time ( day 8) and temperature (30 °C). The tetracycline produced by *Streptomyces remosus* NCIM 2213 in accordance with the present method, is free of undesirable effects that is advantageous for its use in pharmaceutical industries. From the results of the present study, it may be concluded that fruit processing solid residues can serve as a substrate for the production of some value added product using SSF. The use of fruit waste for the production of tetracycline may have the combined benefit of utilizing a low-grade waste while producing a commercially valuable product.

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