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Optimization and Production of Neomycin from Different Agro Industrial Wastes in Solid State Fermentation

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ABSTRACT
The cost of neomycin production may be significantly decreased by using inexpensive carbon substrates like agricultural residues. However, scarce information can be found in the literature about the utilization of cellulosic and lignocellulosic residues for obtaining neomycin. Usually agricultural residues producing various toxic compounds to the atmosphere; so, as an interesting alternative to the utilization of agricultural wastes (as apple pomace, cotton seed meal, soy bean powder and wheat bran) for simultaneous neomycin production. The highest neomycin production (2765 µg/g substrate) was achieved with apple pomace in solid-state fermentation. The optimization of physical parameters such as inoculum size, substrate particle size, incubation temperature, initial pH, initial moisture level, incubation period and chemical parameters such as additional carbon and nitrogen sources were studied for the production of neomycin in solid-state fermentation using *Streptomyces fradiae* NCIM *2418*. The optimum values of the critical components determined for the maximum neomycin production were inoculum size 2×10^6 CFU/g initial dry substrate, substrate particle size 1.2 mm, incubation temperature 30°C, initial pH 8, initial moisture level 70%, fructose (1% w/v), $(NH_4)_2HPO_4$ (1% w/v), L-glutamine (1%w/v) and incubation period day 10. An overall 2.6-fold improvement in neomycin production was achieved due to optimization.

Keywords: Solid-state fermentation, *Streptomyces fradiae* NCIM *2418*, Optimization, Neomycin.

INTRODUCTION

Neomycin is an important aminoglycoside antibiotic, which is effective against gram-positive and gram-negative bacteria, and mycobacteria. Many microorganisms have been evaluated for the production of neomycin including *streptomyces* strains such as *Streptomyces marinensi*, [1] *Streptomyces fradiae.* [2] However, high cost and low yields of neomycin have been the main problems for its industrial production. [3] Therefore, there is a great need to develop a new fermentation medium with inexpensive substrates that provides a high neomycin yield.

It is well known that 30-40% of the production cost of antibiotics is taken up by the cost of growth medium. [4] Carbon and nitrogen sources together with fermentation time have been reported to play significant roles in the determination of the final morphology of the culture. [5]

Among existing technologies in the fermentation industry, solid-state fermentation (SSF) shows many advantages over fermentation with submerged culture, such as lower cost and much higher reactor volume. [6] The application of SSF process has a considerable economical potential in the food, feed, pharmaceutical, and agricultural industries. There are a great number of literatures reported to use the SSF process for producing antibiotics with industrial importance, such as penicillin, oxytetracycline, tetracycline, cephamycin C, cephalosporin C, meroparamycin, rifamycin B, neomycin, iturin A and tylosin. [7-16] However, it has not been reported using the SSF for production of neomycin using apple pomace and cotton seed meal.

India has the largest production of apple, cotton, soy bean and wheat in the world. Millions of tons of apple pomace, cotton seed meal, soy bean powder and whet are also produced each year. In 2009, the yields of apple pomace and cotton seed meal were about 1 and 6 million tons, respectively. These relatively cheap agro industrial residues, containing abundant nutrients (hemicelluloses cellulose, proteins and starch), have a great potential to be utilized as alternative fermentation substrates. Therefore, in this research, apple pomace, cotton seed meal, soy bean powder and wheat bran were selected and used as basic carbon and nitrogen sources for production of neomycin.

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In the present study, the productivity of neomycin by *Streptomyces fradiae 2418* using solid agro-industrial residues such as apple pomace, cotton seed meal, soy bean powder and wheat bran was evaluated. In addition, the culture conditions as initial moisture content, initial pH, inoculum size, incubation temperature, and substrate particle size as well as the extra supplementation of carbon and nitrogen sources were optimized to maximize the antibiotic yield. To the best of our knowledge, there is no reported publications deal with the production of neomycin by *Streptomyces fradiae 2418* under solid state fermentation.

MATERIAL AND METHODS

Culture media and culture condition

Streptomyces fradiae NCIM *2418* was the actinomycetes used in the present study. The strain was provided by National Collection of Industrial Microorganisms, Pune, India. The spores of this strain had been preserved in a dormant state in potato agar slants. The spores were revived to an active state of growth by transferring to the following growth medium (g l^{-1}): glucose 4, yeast extract 5, peptone 10.Inocula were prepared by transferring a loopful of seed culture into 50 ml of inoculums medium in a 250- ml Erlenmeyer flask. The composition of the inoculum medium was the following (g l^{-1}): soluble starch 20, tryptone soy broth 20, yeast extract 3, $CaCO_3$ 3, K_2HPO_4 1, $MgSO_4.7H_2O$ 0.025, and pH were adjusted to 7.2. The flasks were incubated on a rotary shaker at 170 rev min^{-1}at 30°C. After 3 days, the whole culture was harvested by centrifugation at 7826 g for 10 min, and the cell pellet was washed thoroughly with saline solution. *Staphylococcus epidermidis* NCIM 2493 [17] was used an indicator organism.

Substrates

Apple pomace was obtained from a local apple juice concentrate company in Dharwad, India. It was dried in an oven at 60°C and ground in a hammer mill. The ground material was passed through 30- and 50-mesh sieves. The fraction which passed through the 30-mesh sieve but retained by the 50-mesh sieve was collected and used as basic fermentation media. The cottonseed meal was obtained from a local market at Dharwad, India. The meal was made after cotton seed oil extraction using a compression method and was pre-treated in the same way as the apple pomace. Soy bean powder and wheat bran obtained from local market was pre treated as same as for the apple pomace.

Solid state fermentation

Ten grams of solid substrate, in a 250 ml Erlenmeyer flask, were moistened with mineral salt solution (g l^{-1}: KH_2PO_4, 1; $MgSO_4$, 0.4; pH 7.0), thoroughly mixed and autoclaved at 121°C for 30 min. The cooled medium was inoculated with 48 h old inoculum (2.0×10^6 CFU/g initial dry substrate) and incubated at 30°C for 5 days. The moisture content of the medium after inoculation was 50%. Unless otherwise specified, these fermentation conditions were maintained throughout the experiment.

Measurement of pH and Moisture Content

The pH was determined using 1.0 g of fermented material in 10 ml of distilled water, and then the mixture was agitated. After 10 min, the pH was measured in the supernatant using a pH meter. The moisture content of the medium was estimated by drying 5 g of the wet sample to a constant weight at 105°C and the dry weight was recorded. [18]

Antibiotic extraction

At the end of fermentation, the harvested biomass was treated with 50 ml of phosphate buffer and agitated thoroughly on a magnetic shaker for 30 min. The whole contents were filtered through sterile muslin cloth, and residues were again treated with another aliquot of 50 ml of phosphate buffer as previously and subsequently filtered. The filtrates were pooled then centrifuged, and the final clear supernatant was used as the antibiotic source.

Antibiotic assay

The disc diffusion bioassay method that utilizes the antibacterial property of ne neomycin to produce a zone of inhibition against *Staphylococcus epidermidis* was used. The method employed the use of filter paper discs containing 10 µl of supernatant from fermentation broth of *Streptomyces fradiae* NCIM *2418* and negative control. These discs were dried and placed on the surface of agar plates seeded with spores of *Staphylococcus epidermidis* NCIM 2493 strain. Positive control was consisted of disc with known amount of neomycin. These plates were incubated at 37°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 micro grams of neomycin produced per gram of substrates. [19]

Optimization of the culture condition for neomycin production

The different physicochemical parameters to maximize the yield of neomycin by *Streptomyces fradiae* NCIM *2418* under solid state fermentation were investigated. The optimized parameter was incorporated at its optimized level in the subsequent optimization experiments. The impact of initial moisture content (20-90%), initial pH (3-11, adjusted with 1N HCl or 1N NaOH), incubation temperature (20-40°C), incubation period (4-12 days), particle size and size of inoculum on neomycin production using solid state fermentation *of Streptomyces fradiae* NCIM *2418* was evaluated. Moreover, the effect of incorporation of additional carbon sources (glucose, arabinose, mannitol, sorbitol, and fructose at 1%w/v), additional nitrogenous compounds ($NaNO_3$, NH_4Cl, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ yeast extract, casein, beef extract, L-asparagine, L-methionine, malt extract and L-glutamine at 1% w/v), to the production medium were studied. All the experiments were conducted in triplicate and the mean values are considered. After incubation of each fermentation sample, the crude extract was prepared.

RESULT AND DISCUSSION

Evaluation of different agro-industrial material for neomycin production

The fermentation profile of neomycin production in SSF varied with type of agro material used. Highest antibiotic production (4567 µg/g substrate) was observed with apple pomace and the least (2765 µg/g substrate) with wheat bran. A 2-fold variation was noticed with these materials (Fig 1). This could be attributed to solid materials dual role-supply of nutrients and anchorage to the growing microbial culture which influence the microbial growth and subsequent metabolite production. Such substrate dependent microbial product yield variations were also reported in literature. [20] These results depict that the selection of an ideal agro-biotech source for neomycin production depends primarily on the availability of carbon and nitrogen source and thus screening of several agro-industrial residues is essential.

Ellaiah *et al.,* (2004) [14] working with *Streptomyces. marinensis* NUV 5,reported that wheat bran is the better solid support material for the production of neomycin under SSF. However, in the present study, among all studied materials, wheat bran supported least production of neomycin production. This may be attributed to the fact that the strains used by them may vary in their metabolic pattern compared to *Streptomyces fradiae* NCIM 2418 used in the present study or carbon source material associated with wheat bran may not be utilized by the *Streptomyces fradiae* NCIM 2418. To evaluate the same, the hemicelluloses and cellulose hydrolysis ability of the strain was investigated. This data further confirm that high antibiotic titers associated with apple pomace were due to the maximum production of hemicelluloses and cellulose hydrolyzing enzyme by the strain and as strain *Streptomyces fradiae* NCIM *2418* is hemicellulases positive hence could utilize hemicelluloses as carbon source. Hence, it could be concluded that the selected strain requires substrates that provide hemicelluloses as its enzymatic machinery that hydrolyzes the polysaccharides present in substrates.

Effect of substrate particle size

In solid-state fermentation process, the availability of surface area play a vital role for microbial attachment, mass transfer of various nutrients and substrates and subsequent growth of microbial strain and product production. The availability of surface area in turn depends on the particle size of the substrate/support matrix. The experimental data revealed that neomycin production was affected by the particle size. Maximum antibiotic production (4567 µg/g substrate) was noticed with 1.2 mm substrate particle size green gram husk material (Fig 4). Altering the substrate particle size in either side of this resulted in reduction of neomycin production. The observed reduction of neomycin production with altered particle size could be attributed to intra-particulate associated aeration, available surface area for microbial attachment and substrate mass transfer and subsequent growth and antibiotic production. These results are in accordance with the literature data on particle size-mediated influence on microbial antibiotic production in *S. marinensis Nuv5* [14] and in *Amycolatopsis sp. RSP 3*. [13]

Effect of moisture level

The moisture level in the solid-state fermentation critically affects the process due to its interference in the physical properties of the solid particle. Increased moisture is believed to reduce the porosity of substrate [21], thus limiting the oxygen transfer. [22-23] The decreased moisture content cause lower availability of media nutrients to the *Streptomyceats* [14] resulting into lower extent of production. Figure 5 shows the effect of total moisture content on neomycin production for 20%, 30%, 40%, 50%, 60%, 70%, 80% and 90% moisture. The result indicated that 70% moisture gave the higher neomycin production during fermentation compared to other treatments. The maximum yield of neomycin production (5356 µg/g substrate) was obtained from 70% moisture at day 8. The results from the previous study stated that the ideal moisture content was 80% and the reduction in antibiotic yield could occur with low and to higher moisture level. [14]

Effect of initial pH of the medium on antibiotic production

The initial pH of the fermentation media may change during fermentation because the substrates employed in SSF usually have the least buffering. Some samples from the fermented mass were aseptically withdrawn, homogenized and pH was checked. The pH of the medium during fermentation was found to be between 3.0 and 11.0, i.e. around acidic to alkaline condition. The initial pH is another important factor which affects the growth and antibiotic production during solid-state fermentation. [24] Substrate was adjusted to different initial pH using 1N HCl and 1N NaOH prior to inoculation. Fig. 3 shows that a good neomycin production (5923 µg/g substrate) was observed at pH 8.0.

Effect of temperature

The maintenance of an optimal process temperature is one of the major factors in the economics of a process. Temperature affects microbial cellular growth, spore formation, germination and microbial physiology, thus affecting product formation in turn. 30^{o}C was found to be the optimum temperature in this case (Fig. 5). Ellaiah *et al.,* (2004) [14] reported an optimum temperature of 30^{o}C for neomycin production by SSF using *Streptomyces. marinensis NUV 5*, while Howard TD (1952) [2] reported 28^{o}C as optimum for neomycin production by submerged fermentation (SmF) using *Streptomyces Fradiae*.

Effect of inoculum size

The optimum inoculum size for neomycin production (4673 µg/g substrate) by *Streptomyces fradiae* NCIM *2418* was 2×10^{6} CFU/g initial dry substrate (Fig. 6). Adequate inoculum can initiate fast mycelium growth and product formation, thereby reducing the growth of contaminants. A decrease in antibiotic production was observed when the inoculums size was increased beyond the optimum level. Antibiotic production attains its peak when sufficient nutrients are available to the biomass. Conditions with a misbalance between nutrients and proliferating biomass result in decreased antibiotic synthesis. [13]

Effect of incubation period

Solid-state process was performed for various incubation periods. Remarkably higher levels of neomycin production were observed following 6–10 days of the process and maximal levels (6453 µg/g substrate)) was achieved at the 12th day of fermentation (Fig.7). Important ascend in neomycin yield with increased biomass was observed during 8th–10th days of fermentation cycle. Significant variation in neomycin production was observed during different fermentation periods.

Effect of nitrogen source on neomycin production

Generally, the high concentration of nitrogen sources in media is effective in enhancing the production of neomycin by *Streptomyces fradiae.* [2, 25] The protein content in apple pomace is very low so that the nitrogen levels as well as the commercial value all decrease greatly. [26] Hence, the exogenous addition of various nitrogen levels to the solid medium was studied.

Effect of supplementation using different inorganic and organic nitrogen sources on the production of neomycin is illustrated in Fig. 8 and Fig 9. $NaNO_3$, NH_4Cl, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ as inorganic and yeast extract, casein, beef extract, L-asparagine, L-methionine, malt extract and L-glutamine was used as complex organic nitrogen source, for supplementation of additional nitrogen. Based on the results it was found that L-glutamine was the best organic nitrogen source and its supplementation led to further increase in neomycin production to 7432 µg/g substrate (Fig. 9), while

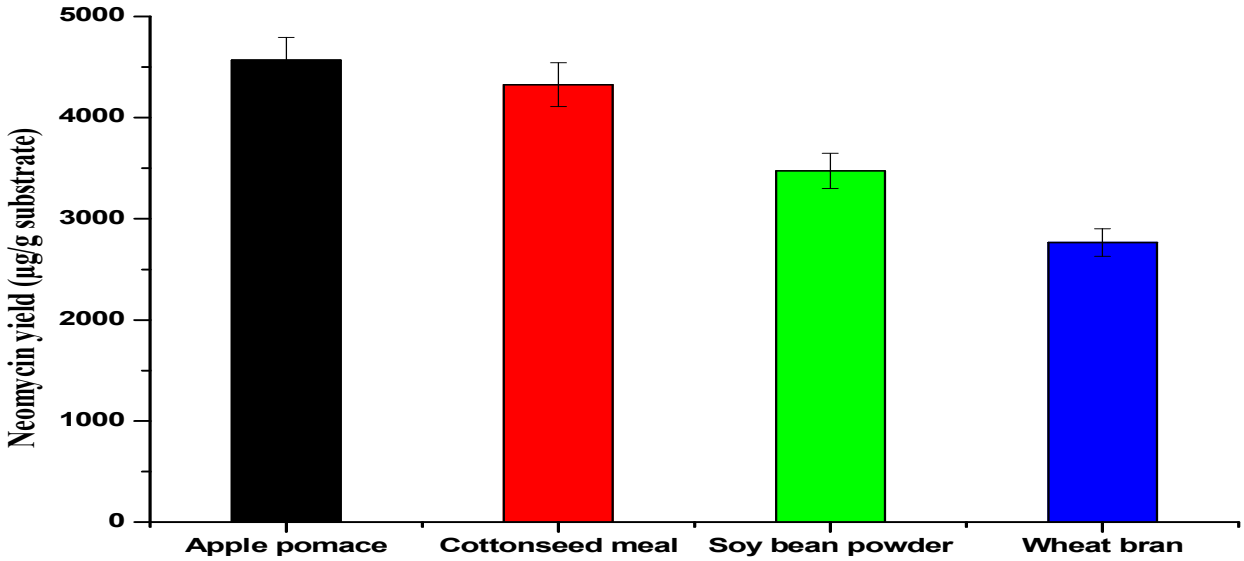

Fig.1: Effect of various substrates on neomycin production by *Streptomyces fradiae* NCIM 2418 under SSF.

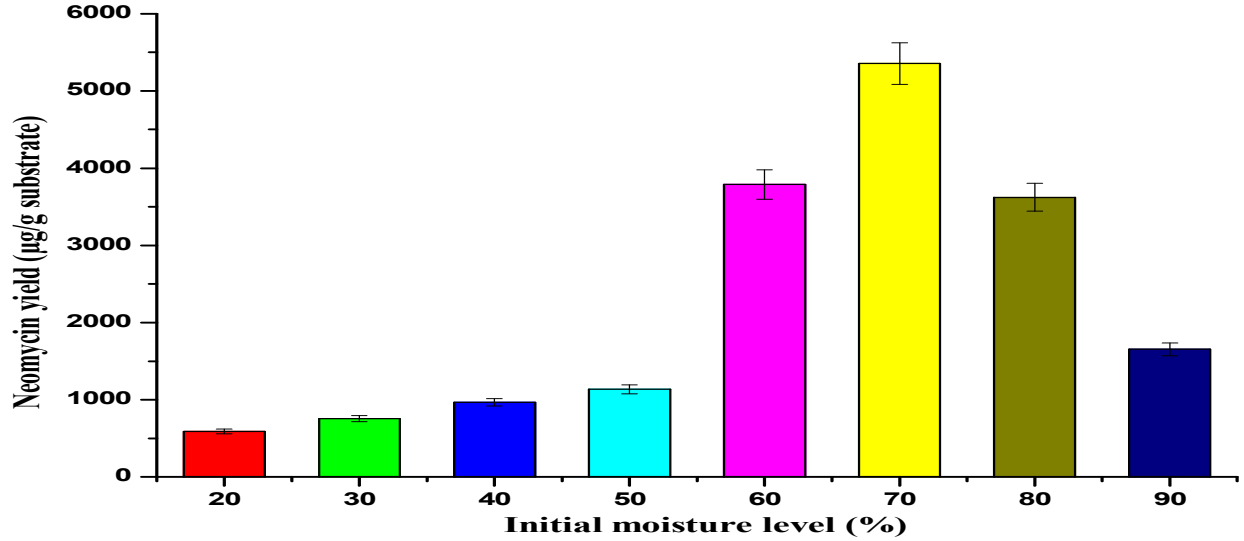

Fig. 2: Effect of various moisture level (%) on neomycin production by *Streptomyces fradiae* NCIM 2418 under SSF.

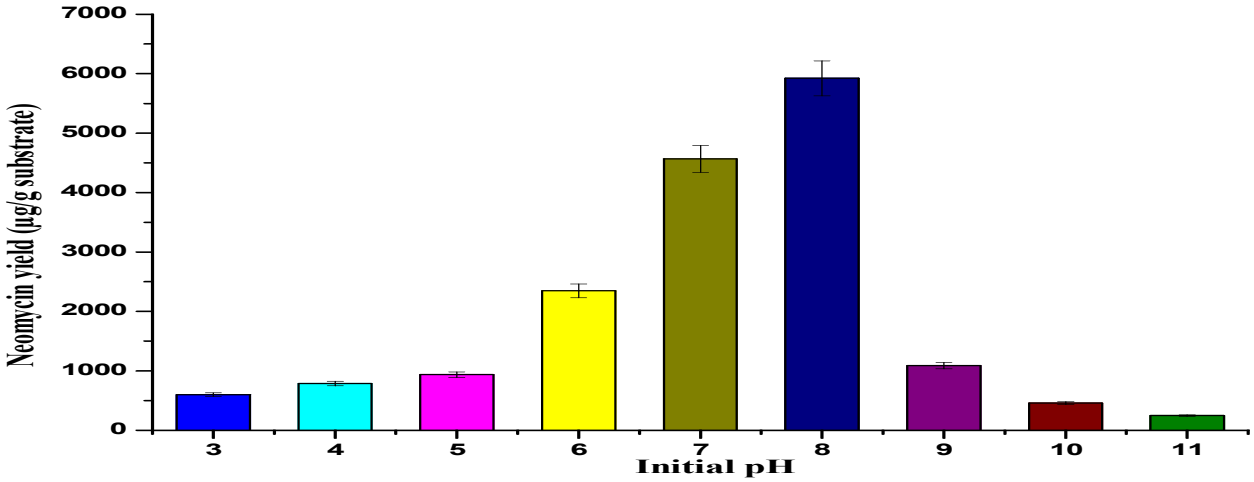

Fig. 3: Effect of various initial pH on neomycin production by *Streptomyces fradiae* NCIM 2418 under SSF.

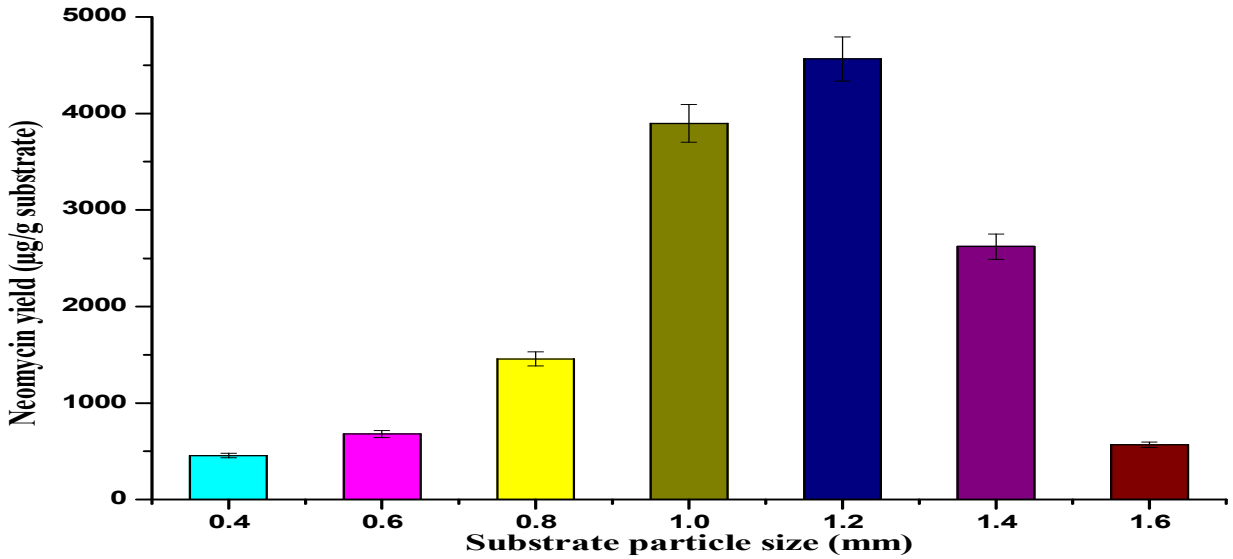

Fig.4: Effect of various substrate partical size on neomycin production by *Streptomyces fradiae* NCIM 2418 under SSF.

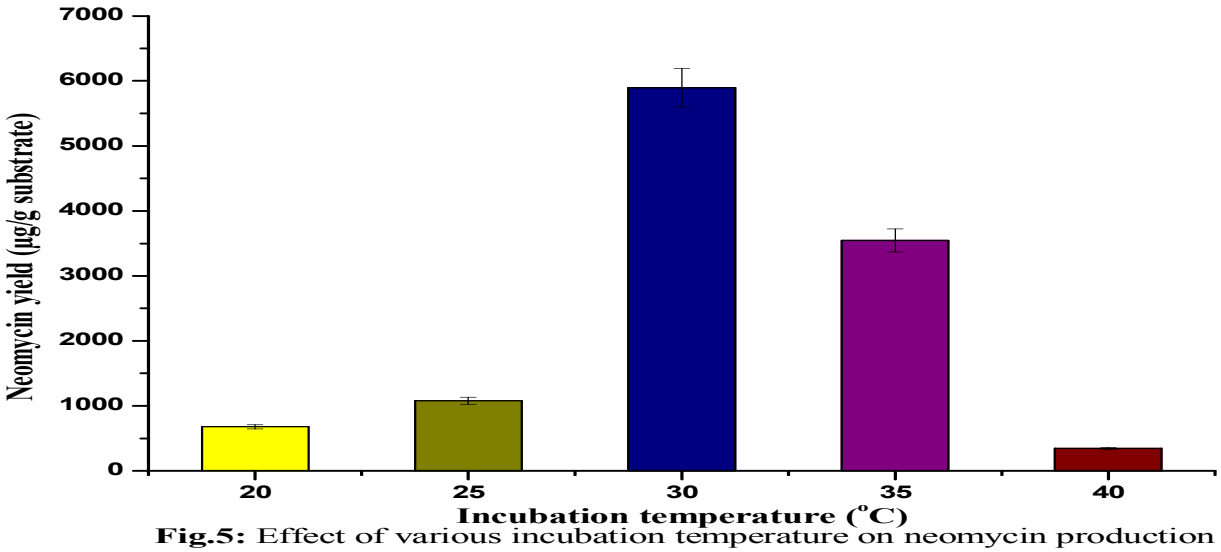

Fig.5: Effect of various incubation temperature on neomycin production by *Streptomyces fradiae* NCIM 2418 under SSF.

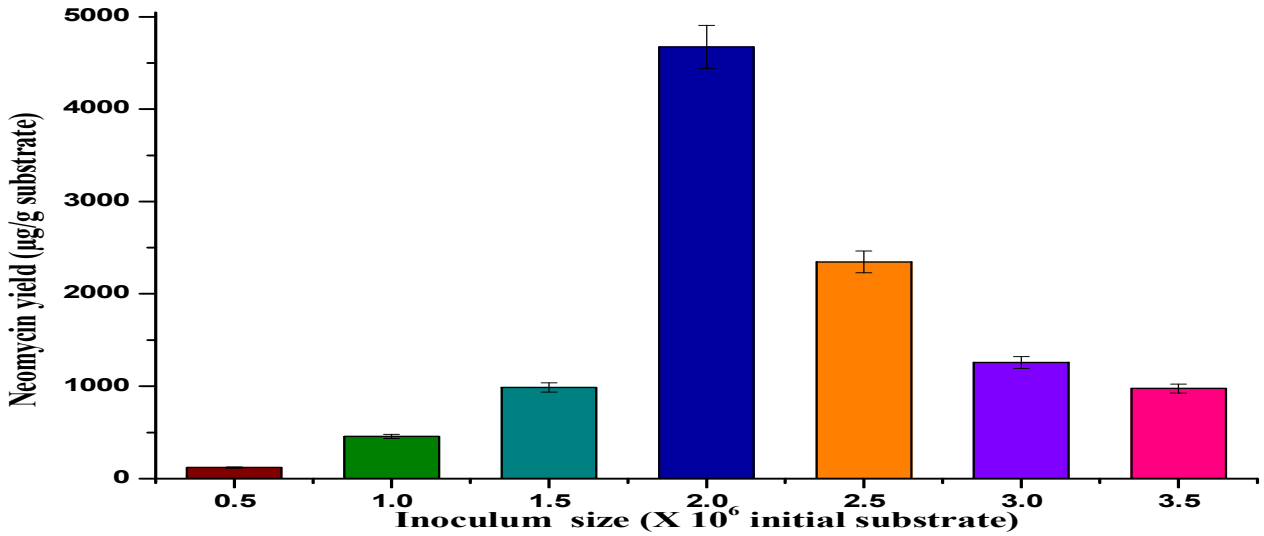

Fig. 6 : Effect of inoculum size on neomycin production by *Streptomyces fradiae* NCIM 2418 under SSF.

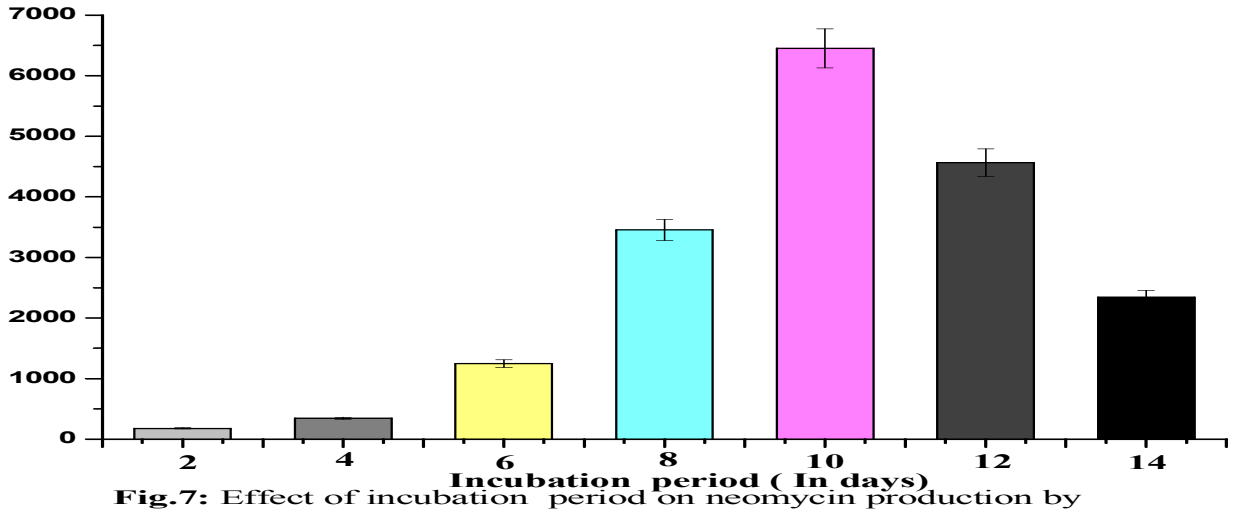

Fig.7: Effect of incubation period on neomycin production by *Streptomyces fradiae* NCIM 2418 under SSF.

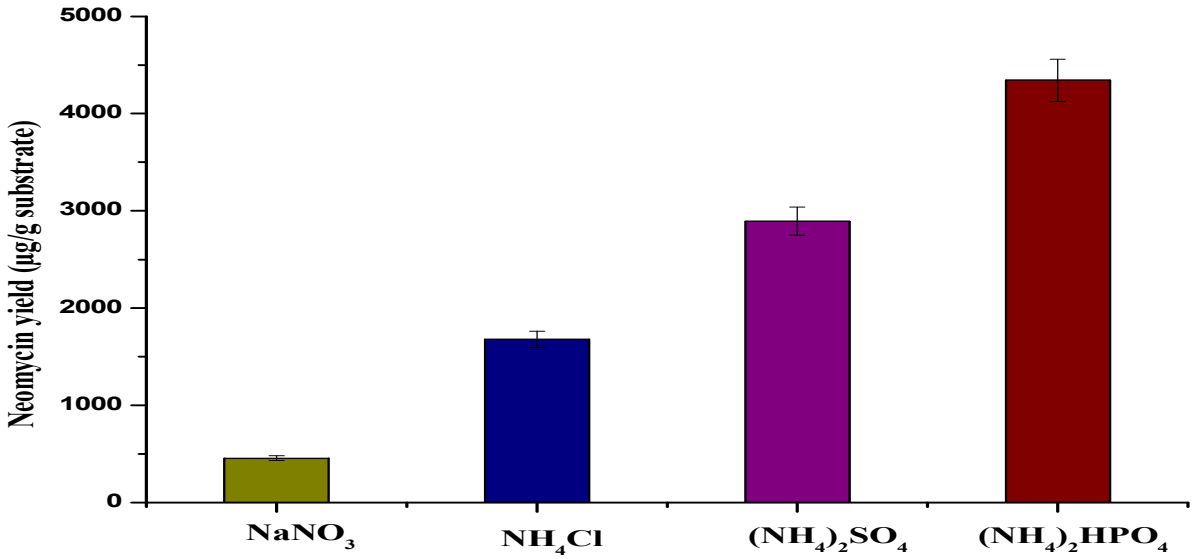

Fig. 8: Effect of different inorganic nitrogen sources (1% w/v) on neomycin production by Streptomyces fradiae NCIM 2418 under SSF.

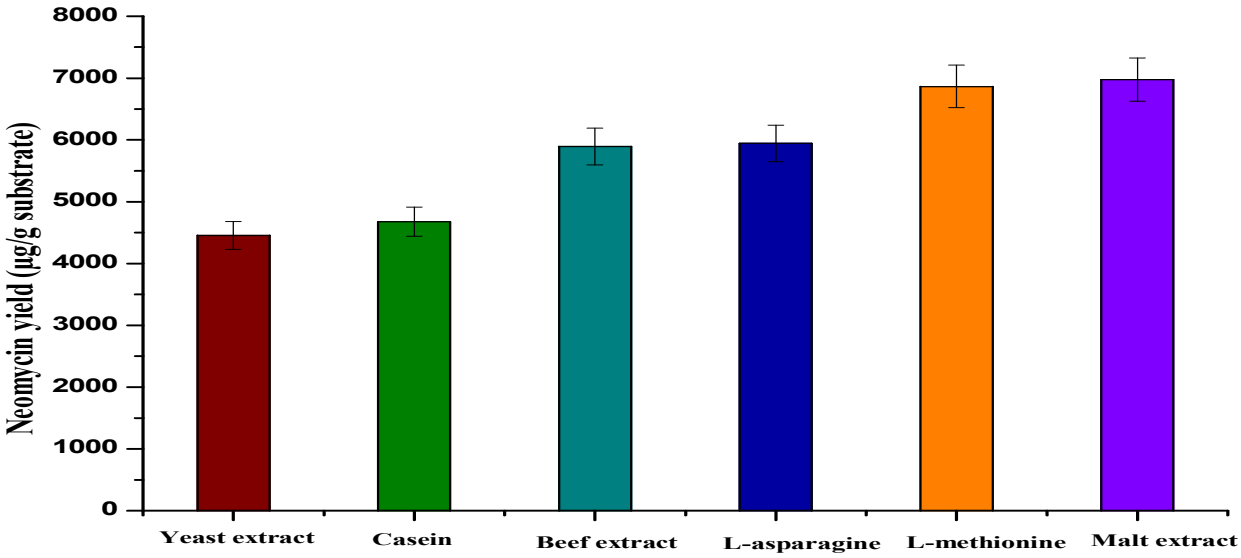

Fig. 9: Effect of different organic nitrogen sources (1% w/v) on neomycin production by *Streptomyces fradiae* NCIM 2418 under SSF.

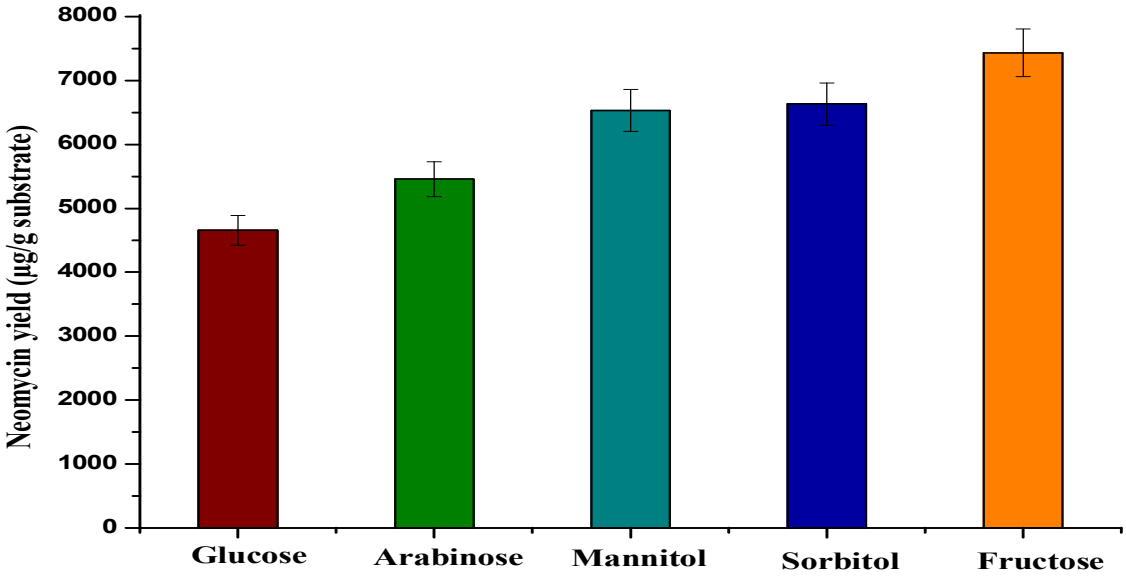

Fig. 10: Effect of different carbon sources (1% w/v) on neomycin production by Streptomyces fradiae NCIM 2418 under SSF.

inorganic nitrogen source such as $(NH_4)_2HPO_4$ was found best neomycin production (4342 µg/g substrate) (Fig. 8).

Effect of carbon source on neomycin production

Although apple pomace can support the growth of *Streptomyces fradiae* NCIM *2418* and neomycin production, it may not provide enough carbon sources needed [27] by the organism for maximum antibiotic production. Hence, the exogenous addition of various carbon sources to the medium may improve cell growth and antibiotic production. Generally, the high concentration of carbon sources in media is effective in enhancing the production of neomycin by microorganisms. [2, 25]

The impact of supplementation of external carbon sources on neomycin production was studied and the results were shown in Fig 10. Addition of carbon sources with 1% w/v concentration to the medium showed different effects on neomycin production. The *Streptomyces fradiae* NCIM *2418* was grown on the medium with carbon source more rapidly at the first 8-10 days, but it turn to autolysis soon, resulted in less mycelia and then less neomycin at day 12. So among all the compounds tested, fructose yielded the highest neomycin production (7432 µg/g substrate), followed by sorbitol (6632 µg/g substrate), mannitol (6532 µg/g substrate), arabinose (5456 µg/g substrate) and glucose (2.17 µg/g substrate). So fructose and sorbitol can be added as supplementation of carbon sources in basal substrate to prolong cell growth and/or to improve neomycin secretion.

Results obtained in this study indicated that among the various agro-industrial residues studied, apple pomace was the suitable substrate for neomycin synthesis by *Streptomyces fradiae* NCIM *2418* in SSF. SSF showed its superiority for antibiotic production and also revealed the possibilities of effective utilization of apple pomace (and possibly other agro industrial residues) for value addition through biotechnological means. The optimal conditions for neomycin production using SSF for apple pomace initial pH (8), initial moisture level (70%), substrate particle size (1.2 mm), inoculums size (2 x 10^6 CFU/g), incubation temperature(30^{o}C), incubation period (day 10), fructose (1% w/v), $(NH_4)_2HPO_4$ (1% w/v), L-lutamine (1%w/v) respectively. Such processes would not only help in reducing the cost of production but also pave the way in effective solid waste management. With the above encouraging leads, it will be interesting to study the apple pomace as substrate for the production of other antibiotics from different microbes.

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