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ASSESSMENT OF EFFECTS OF FERMENTATION DURATION AND P^H FOR OPTIMAL ANTIBIOTIC PRODUCTION BY STREPTOMYCES SPECIES ISOLATED FROM NIGERIA SOIL

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ABSTRACT

Submerged fermentation of batch cultures of broad spectrum antibiotics producing *Streptomyces* isolates from Uyo, Nigeria in basal secondary fermentation medium ISP 2, were subjected to time-course study for assessments of effects of fermentation duration for and effect of P^H (6.0 – 12.0) for 14 days. Daily antibiotic titre were assessed by inhibitory activity index using by the agar-well diffusion technique on DSTA seeded plates of *Staphylococcus aureus* and spectrophotometrically by measuring absorbance at 230 nm. Time-course study showed that daily antibiotic production titre significantly ($p< 0.05$), correlated positively with sporulation ($r =0.805$), increasing steadily to maximum peaks of inhibitory activity and absorbances at the following optimal durations: UY4 (7^{th} days: 36.0 mm, 0.049 nm), UY5 (9^{th} day: 39.0 mm, 0.079 nm), UY7 (6^{th} day: 38.0 mm, 0.054 nm), UY8 (8^{th} day: 240 mm, 0.059 nm), (7^{th} day: 36.0 mm, 0.049 nm), UY9 (7^{th} day: 34.0 mm, 0.054 nm), before gradually decreasing with further incubation within the study period. Similarly, optimal pH for antibiotic production by the isolates were within the range 8.0 – 9.0 as follows UY4 (pH 8.0, 18.0 mm), UY5 (pH 9.0, 19.0 mm), UY7 (pH 8.5, 23.0 mm), UY8 (pH 8.5, 16.0 mm), UY9 (pH 8.0, 20.0 mm), suggesting the *Streptomyces* isolates to be alkilophiles. The results indicated that the optimal fermentation duration and pH for growth and antibiotic production by the *Streptomyces* isolates are species-specific.

KEYWORDS: Assessment, Fermentation Duration, pH, Optimal Antibiotic Production, *Streptomyces* species.

INTRODUCTION

The filamentous soil bacteria belonging to the genus *Streptomyces* are widely recognized as industrially important microorganisms, because of their ability to produce many kinds of novel secondary products, including diverse antibiotics (Crandall and Hamil, 1986; Watve, *et al.*, 2001). The genus *Streptomyces* was proposed by Waksman and Henrici for the aerobic and spore-forming Gram-positive Actinomycetes having a deoxyribonuclei acid, with a high guanosine and cystocine (G+C) content of 69 – 73 mol %, with an extensive branching substrates and aerial mycelia (Williams *et al.*, 1983a; Williams *et al.*, 1989). The genus *Streptomyces* is the largest and most important antibiotics producers in the microbial world so far discovered. Indeed, bacterial species in this genus account for the exponential increase in the number of antibiotics per year isolated and characterized in the past decades. (Crandall and Hamil, 1986; Tomita *et al.*, 1989; Miyadia, 1993; Hassan *et al.*, 2001; Watve *et.al*, 2001; Sahin an Ugar, 2003,). Also recent reports showed that this group of microorganisms still remains an important source of screening for new antibiotics (Augustine *et al.*, 2005; Jain and Jain, 2005; Sujatha, 2005; El-Naggar, 2007; Ceylan *et al.*, 2008; Atta *et al.*, 2009; Atta, 2010; Scheeviamni *et al.*, 2010; Atta *et al.*, 2011; Krishnaveni *et al.*, 2011; Abdelghann, 2011; Ekong *et al.*,2013, Ekong *et al.*, 2016). The growth and optimal production of secondary metabolite by microorganisms, including antibiotics are highly dependent upon the surrounding cultural conditions, which are influenced by many factors, mainly physiological and genetical (Sermonti, 1969; Martin, 1978; Martin and Demain, 1980; Demain, 1998, Bibb, 1996; Hopewood, 1999; Bibb, 2005). Every organism has a range of ideal cultural conditions including fermentation duration and pH at which members of its species will grow and optimally produce secondary metabolites. Hence, the ability of *Streptomyces* cultures to produce antibiotics is not a fixed properly, but can be greatly increased or completely lost under different cultural conditions such as reduced or prolonged fermentation duration

(Abdelghani, 2011) and influence of pH (Konto, *et al*, 2005). Thus, there is a paucity of information on the effects of fermentation duration and pH on antibiotic production by *Streptomyces* isolates from Nigeria soil. Therefore, this research work investigates the effects of fermentation duration and pH, and as well reports their optimal conditions for growth and antibiotics production by the *Streptomyces* species isolated from Nigeria soil.

MATERIALS AND METHODS

Antibiotics Producing Cultures

The antibiotics producing *Streptomyces* cultures were isolated from pre-treated soil samples from Uyo, Nigeria Actinomycetes medium, ISP1 by the crowded-plate technique, and confirmed by an overnight bioautography against 0.5 MacFarland nephelometer standardized broth culture of *Staphylococcus aureus* (Ekong, *et. al.*, 2013).

Submerged Fermentation, Antibiotic Production and Activity

Antibiotic producing *Streptomyces* isolates in 1.0ml spore-suspension were inoculated in 50.0 ml of basal secondary fermentation medium, (ISP 2) of Sahin and Ugur (2003), of the following composition (g/L); NaCL, 1.0; NH_4Cl, 1.0; KCL, 1.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 2.0; $CaCl.2H_2O$, 0.04; glucose, 2.0; yeast extract, 3.0; distilled water, 1 litre). The submerged cells were subjected to fermentation in an orbital-shaker incubator (120 r.p.m, 28 ^oC, 5 days). The submerge cells were subjected to fermentation, as described by Ekong *et al.*, (2015); Ekong and Ibezim (2016). At the end of fermentation, cultures were harvested and subjected to centrifugation (4000 x g, 30 mins, 4 ^oC) followed by aseptical filtration of the supernatants through Gelman acrodisc filter (0.45 µm pore diameters). The resultant crude antibiotic solutions were stored at 4 ^oC for all subsequent antibiotic activity assays (Ekong *et al.*, 2004). Antibiotic activity was assessed on diagnostic sensitivity test agar (DSTA) seeded plates of *Staphylococcus aureus*, by the modified agar-well diffusion technique and spectrophotometrically by measuring the absorbances at 230 nm (Ekong *et al.*, 2004).

Effect of Fermentation Duration on Antibiotic Production

Time course studies of the effect of fermentation duration on antibiotic production were carried out in 50ml of ISP2 under fermentation conditions and to those employed to produce the antibiotics solutions for a period of 14 days. Samples of the fermentation broths were daily harvested, processed and tested for antibiotic activity as previously described. Thereafter, a graph of daily zones of inhibition and absorbances against time were plotted, and the optimal fermentation duration for antibiotic production by the respective isolates determined.

Effect of pH on Antibiotics Production

The effect of pH on antibiotic production by the *Streptomyces* isolates was investigated in 50ml of pH readjusted basal ISP2 medium within the pH range (6.0 – 12.0) under similar fermentation conditions obtained in the production of the antibiotic solutions at the respective time course optima for the different isolates. After fermentation, sample broths were harvested, processed and tested for antibiotic activity as previously described. The pH dependent zones of inhibitory activity and absorbances produced by the isolates were plotted, and the optimal pH determined for the isolates.

RESULTS AND DISCUSSION

Effect of Fermentation Duration

Time course study revealed the progressive release of appreciable antibiotic substances by the sporulating *Streptomyces* isolates into the fermentation medium within the study duration. antibiotic titres produced by the isolates reached maximal peaks at the following fermentation duration at sporulation: UY 4 (7^{th} day; 36.0 mm, 0.049 nm), UY5 (9^{th} day; 39.0 mm, 0.079 nm), UY7 (6^{th} day; 38.0 mm 0.055 nm), *Streptomyces species* UY8 (8^{th} day; 24.0 mm, 0.059 nm) *Streptomyces* sp. UY9 (7^{th} day; 34.0 mm, 0.055 nm); and thereafter decreased with increase in fermentation period (Fig. 1). The result indicated lack of or low antibiotic titre in non-sporulating cultures, but significantly ($p< 0.05$) present in sporulating cultures which marked entry into idiophase, and the beginning of secondary metabolite production. These changes in antibiotic titres showed significant ($p< 0.05$) positive correlation with sporulation ($r = 0.805$), suggesting that sporulation plays a significant role in antibiotic –biosynthesis in the idiosphase. This is obvious in that with prolonged fermentation, antibiotic-titres, decreases, suggesting that the sporulating cultures entered the decline (death) phase, primarily due to nutrient exhaustion and accumulating levels of toxic antimetabolic antimbiotic titres. This observation agrees with an earlier report that antibiotic titres in fermentation broth sharply decreased with prolonged fermentation period in sporulating cultures due to cell autolysis caused by nutrients exhaustion (Yang and Yuan, 1990, Yang and Swei, 1996; Vastrad and Neelagund, 2011). In the respective cultures, antibiotics production was virtually absent or very low in the rapidly growing cultures, but only appeared in the sporulating cultures at idiophase, which is marked by secondary metabolites production. The results are in line with that reported for other filamentous and spore-forming organisms (Bhattacharyya *et al.*, 1998; Hassan *et al.*, 2001). The enhanced, antibiotic substances production by sporulating cultures in idiophase, has been linked to the expression of antibiotic biosynthetic genes as a result of the physiological changes taking phase in the idiophase (Martin and Demain 1980; Gramaja *et al.*, 1993; Hopewood, 1999; Hassan *et al.*, 2001). Nevertheless, in the study, the growth and optimal antibiotics production by the isolates, which occurred within the fermentation duration range of 6 – 9 days, explicitly corresponded

with sporulation and entry into idiophase, thereby supporting the widely reported fermentation duration range of 4 – 9 days for other *Streptomyces* species (Yang and Swei, 1996; Augustine *et al.*, 2005; Knshaveni *et al.*, 2011; Vastrad and Neelagund, 2011). The observed fermentation duration by the *Streptomyces* isolates in the study, could be linked with their ability to either rapidly or slowly utilize the nutrients in the fermentation medium for both growth and antibiotic production, resulting in the short or long optimal fermentation period. This assertion agrees with the wide reports that *Streptomyces* species that rapidly utilized nutrients in fermentation media, required shorter fermentation period for growth and secondary metabolites production, including antibiotics and vice-versa (Yasuzuwa *et al.*, 1987; Vilches *et al.*, 1990; Haque *et al.*, 1995; Cruz *et al.*, 1999; Adinarayana *et al.*, 2002; Sanchez and Demain, 2002; Tripathi, 2004; Gehshewa Saudagar and Singhal, 2007; Abdel Ghani, 2011).

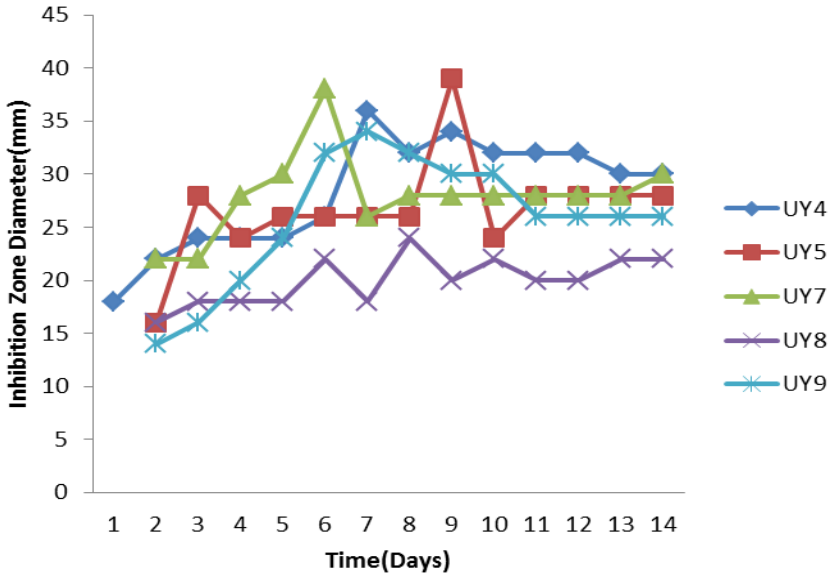

Figure 1: Time-course study of Antibiotic Production by *Streptomyces* isolates.

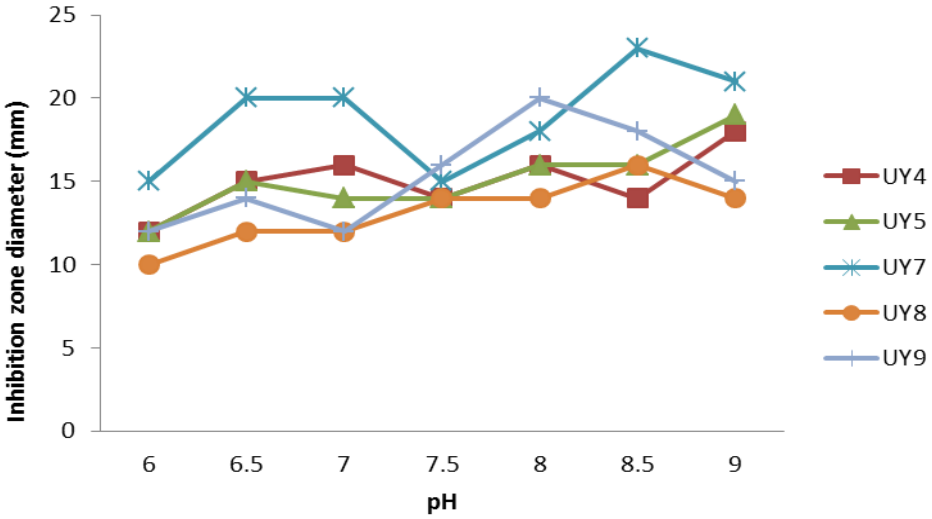

Figure 2: Effect of pH on Antibiotic Production by *Streptomyces* isolates.

The reduction or increment in antibiotic fermentation period by *Streptomyces* species has been reported to be directly linked with the nature of the carbon and nitrogen sources in fermentation media, as well as the ability of isolates to promptly utilize these nutrients in the media for both growth and antibiotic production (Bhatnagar *et al.*, 1988; Hague *et al.*, 1995; Lee, 1997; Abbanat *et al.*, 1999; Cruz *et al.*, 1999; Mohammed, 2000; Adinarayena *et al.*, 2002; Gesheva, 2005; Jakeman *et al.*, 2006; Saudagar and Singhal, 2007; Paul and Benerjee, 2008, Ghosa and Prasad, 2010; Ilic *et al.*, 2010). Hence some media often repressed the growth of *Streptomyces* species, thereby increasing the fermentation period, while others promptly support growth, leading to

increased antibiotic production within a short period. This assertion is corroborated by this study, presenting media effect as the isolates with short fermentation period may indicate the likelihood of prompt nutrient utilization of the ISP 2 medium for both growth and antibiotic production and vice-versa. Thus, the ability and rate of which the *Streptomyces* isolates breakdown the nutritional constituents of the ISP 2 medium, into useful and easily assimilable components could be of utmost-importance in determining their optimal fermentation periods.

Effect of pH on Antibiotic Production

The effect of pH on optimal growth and antibiotic production by the *Streptomyces* isolates indicated an alkilophilic pH range of 8.0 – 9.0 as follows: *Streptomyces sp*. UY4 (pH 9.0, 18.0 mm); *Streptomyces sp.* UY5 (pH 9.0, 19.0 mm) *Streptomyces sp.* UY7 (pH 8.5, 23.0 mm), *Streptomyces sp.* UY8 (pH 8.5, 16.0 mm), *Streptomyces sp* UY 9 (pH 8.0, 18.0 mm) (Fig. 2). In the study, pH as one of the physiological and environmental factors, for growth and antibiotic production, was found to have profound influence on growth and antibiotic production by the *Streptomyces* isolates, as it is similarly and widely reported for other *Streptomyces* species (Bhattacharrya *et al.*, 19998; Hassen *et al.*, 2001; Kontro *et al.*, 2005; Jakeman *et al.*, 2006; Paul and Benerjee, 2008). The achievement of maximum growth and antibiotic production by the *Streptomyces* isolates within the pH range 8 .0 – 9.0 in the study, is in agreement with the wide optimal pH range of 5.5 – 11.5 for growth and antibiotic production reported for many *Streptomyces* species (Kontro *et al.*, 2001). However, other available reports indicated that majority of *Streptomyces species* grow and produce antibiotics at the optimal neutral or weakly basic pH range of 7.0 – 7.5, and accordingly are classified as neutrophiles (Swetzuna and Osajima, 1990; Bystryks *et al.*, 1996; Chakraberty *et al.*, 2005; Kontro *et al.*, 2005). Consequently, in the study, maximum growth and antibiotic production by the *Streptomyces* isolates accured within the optimal pH range of 8.0 – 9.0. Accordingly, these pH ranges suggested the *Streptomyces* as alkilophiles or alkilotolerant species. These assertion and results are in line with the report that *Streptomyces* and other bacterial species are often capable of growth and secondary metabolites production including antibiotics over a large pH range and may be either neutrophiles or alkilophiles. (Kontro *et.al.*, 2005).

CONCLUSION

The influence of fermentation duration and pH on growth and antibiotic production by *Streptomyces* isolates have been highlighted. Fermentation period was determined by sporulation and entry into idiophase for growth and antibiotic production which were determined by the rate of utilization of media nutrients resulting in either short or long fermentation period. Hence, antibiotic-tires were virtually absent or very low in rapidly growing cultures, and were appreciably higher in sporulating cultures at Idiophase. Similarly, effect of pH as a basic physiological factor regulating enzymatic activity for growth and antibiotic production by the isolates have been shown to be pH dependent, and species –specific in the alkilophilic regime.

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