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Research Article

# Kinetics of Avermectin B1b Production by *Streptomyces avermitilis* 41445 UV 45(m)3 in Shake Flask Culture

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**Background:** Use of avermectin B1b as anthelmintic and insecticidal agent has increased to protect soil and for enhanced crop production. Enhanced production of avermectin B1b was obtained from mutant strain of Streptomyces avermitilis 41445.
**Objectives:** Modeling of mutant strain *S. avermitilis* 41445 UV 45(m) 3 growth and avermectin B1b production is therefore required for optimization during fermentation process. Kinetics of intracellular avermectin B1b production was studies in shake flask culture during submerged fermentation.
**Materials and Methods:** Mathematical models based upon Logistic and Piret Equations have been used to investigate the kinetics of avermectin B1b production and substrate utilization from *S. avermitilis* 41445 UV 45(m)3. Effect of carbon sources (glucose, maltose, lactose, potato starch, soluble corn starch, and wheat starch), pH (6.0, 6.5, 7.0, and 7.5), agitation speed (150, 200, and 250 rpm) on microbial growth and product formation were evaluated.
**Results:** Maximum avermectin B1b production (420.02mg.$L^{-1}$ and cell biomass (31.74 g.$L^{-1}$) were obtained in media having potato starch as carbon substrate at adjusted medium pH 7.5 and 250 rpm agitation speed. Maximum specific growth rate ($\mu_{max}$), growth associated avermectin B1b production coefficient ($\alpha$) and non-growth associated avermectin B1b production coefficient ($\beta$) obtained were 0.16$h^{-1}$, 0.0 mg.$g^{-1}$ and 3.5 mg.$g^{-1}$.$h^{-1}$ respectively.
**Conclusions:** From above results we can conclude that avermectin B1b production is non-growth associated process.
*Keywords:* Avermectin B1b, Logistic and piret equations, *Streptomyces avermitilis* 41445 UV 45(m), Submerged fermentation,

## 1. Background

*Streptomyces avermitilis* is a gram positive, aerobic and mesophilic member of *Actinomycetes*. It is characteristically differentiated into branched substrate mycelium and aerial hyphae which are distinguished into long, compact spiral chains becoming more open as culture matures. The culture grows well at 28 °C and 37 °C but not at 50 °C (1) and is specified for production of complex polyketides known as avermectin (2). Avermectins although lack antimicrobial activities however they exhibit broad spectrum of potent anthelmintic and insecticidal properties (3). Substrate used in cultivation medium not only significantly affects production of avermectin from *S. avermitilis* but also the synthesis of certain enzymes used in the sugar metabolism (4). Microbial production of antibiotics is of great economic importance because the industrial fermentations are moving from the traditional approaches towards the simpler and controlled processes. Kinetic models enable the engineers to construct and control microbial process in elegant way (5). Structured and unstructured models taking into account mathematical and experimental designs are commonly used for expression of the microbial process (6). Similar models have been used previously to demonstrate the fundamental basics of microbial growth kinetics and energetic (7)

## 2. Objectives

Due to complex nature of *S. avermitilis*, kinetics of intracellular avermectin B1b production has been studied improperly. Avermectin B1b is the minor component of commercially available abamectin and has been used as anthelmintic and insecticidal agent. In present study, shake flask fermentation has been done to investigate kinetic parameters for *S. avermitilis* 41445 UV 45(m)3 growth, avermectin B1b production and substrate utilization using Logistic and Piret Equations.

## 3. **Materials and Methods**

### *3.1. Microorganism and Culture Maintenance*

*Streptomyces avermitilis* 41445 UV 45(m)3 obtained through UV mutagenesis (8) was used in the present study.

### *3.2. Inoculum Preparation*

Culture from the nutrient agar slants was inoculated into DSMZ specified YMG medium consisted of ($g.L^{-1}$ in distilled water) glucose 4.0g, yeast extract 4.0g, malt extract 10.0g and $CaCO_3$ 2.0g, and used as seed medium. The pH of medium was adjusted at 6.5 before sterilization. After sterilization at $121^0$C for 15 min, the medium pH was finalized at 7.0±2 with the addition of appropriate amount of $CaCO_3$ (3,9). The medium was incubated at 31ºC in the water bath shaker (Eyela, Japan) for 24 h at 150 rpm.

### *3.3. Fermentation Medium*

The production of avermectin B1b from *S. avermitilis* 41445 UV 45(m)3 were studied independently in SM2 growth medium. Fermentation medium was inoculated with 10%v/v inoculum medium and incubated at 31ºC in water bath shaker for 10 days at 150 rpm. The fermentation medium consisted of ($g.L^{-1}$) soluble corn starch 50.0g, KCl 0.1g, NaCl 0.5g, Yeast extract 2.0g, $MgSO_4 7H_2O$ 0.1g, $CaCO_3$ 0.8g and α-amylase 0.1g. The pH of the medium was adjusted at 7.2±0.2. All the experiments were performed in triplicates in shake flasks. The effect of different carbon sources on growth of *S. avermitilis* 41445 UV 45(m)3 and avemrectin B1b production were employed using the same medium. The medium with preferred carbon source (potato starch) was used to investigate the effect of initial culture pH ranging from 6-7.5 on growth *Streptomyces avermitilis* 41445 UV 45(m)3 and avemrectin B1b production. The effect of agitation speed was investigated at initial culture pH of 7.5. All the experiments were performed in triplicates.

### *3.4. Analytical Procedure*

Samples taken at 12 h intervals from the fermentation medium were centrifuged (H-1500FR Japan) at 8000 rpm for 15 min at 4°C. Cell growth and avermectin B1b production were observed after the cell biomass analysis. The cell pellet suspended in normal saline was subjected to spectrophotometer for OD measurement at 600 nm. The dry cell weight was measured by filtering the saline suspension through membrane and drying it at 90°C for 24 h in oven. For extraction of intracellular avermectin B1b from fermentation broth, the biomass was taken and supernatant was discarded. The cell biomass in the form of pellet was mixed with appropriate amount of methanol in pestle and motor and crushed hardly to completely dissolve it. The mixture was then centrifuged for the separation of cell biomass and the supernatant was collected for the analysis of avermectin by HPLC. About 20 µl of each extracted sample was applied into HPLC (LC-2080 Shimadzu) where C18 column (SMA C-18) and Detector (UV Variable Wavelength Detector STD-M20A Shimadzu) has been used for separation of components and individual components were eluted by methanol:acetonitrile (98 : 2 $v.v^{-1}$) at a flow rate of 0.5 $ml.min^{-1}$ with a UV absorbance at 246 nm (7).

### *3.5. Kinetics and Mathematical Models*

The growth of *S. avermitilis* 41445 UV 45(m)3 and production of avermectin B1b were described using logistic and Luedeking-Piret equations respectively which are the simplified batch fermentation kinetic models and have been described earlier (10).

### *3.6. Microbial Growth*

The logistic equation can be described as follow:

$$\frac{dX}{dt} = \left[\mu_{max}\left(\frac{1-X}{X_{max}}\right)\right]X \quad (1)$$

**Substrate Consumption**

$$\frac{-dS}{dt} = \frac{1}{Y_{X/S}}\left(\frac{dX}{dt}\right) + m_s.X \quad (2)$$

**Avermectin B1b production**

$$\frac{dP}{dt} = \alpha\left(\frac{dX}{dt}\right) + \beta X \quad (3)$$

Where

X = the concentration of cell biomass ($g.L^{-1}$)

$X_{max}$ = the maximum concentration of cell biomass ($g.L^{-1}$)

$\mu_{max}$ = maximum specific growth rate ($h^{-1}$)

S = Substrate concentration ($g.L^{-1}$)

$Y_{X/S}$ = Yield coefficient of cells on carbon substrate ($g.g^{-1}$)

$m_s$ = maintenance coefficient ($g.g^{-1}.h^{-1}$)

α = growth associated avermectin B1b production coefficient ($mg.g^{-1}$)

β = non-growth associated avermectin B1b production coefficient ($mg.g^{-1}.h^{-1}$)

t = time of fermentation (h)

The equations 1-3 were fitted to the experimental data by non linear regression using Statistica 7.0 software.

## 4. Results

Comparison of *Streptomyces avermitilis* 41445 UV 45(m) 3 growth, avermectin B1b production and substrate consumption evaluated in SM2 media during

submerged fermentation is shown in **Figure 1**. From **Figure 1**, it is clear that the growth arrived at stationary phase after 72h in SM2 medium with 10.30±0.02 $g.L^{-1}$ cell biomass. The avermectin B1b production was not observed up to 72 h of fermentation and started in stationary phase revealing the process to be non growth associated. After the growth entered the stationary phase, the production of avermectin B1b was started and increased. The production of avermectin B1b was maximum (258.6±0.01) at 10th day of fermentation after then it began to decrease. Substrate which is soluble corn starch in the present case was consumed for growth and avermectin B1b production and completely utilized at the end of fermentation.

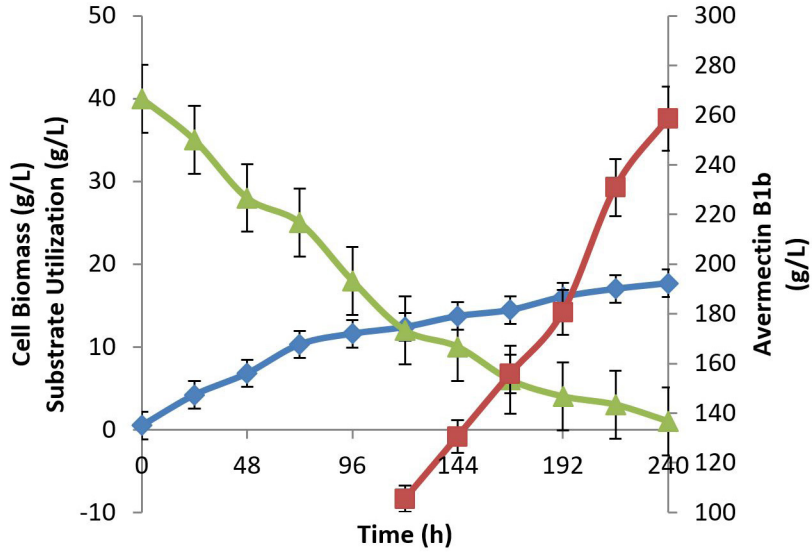


**Figure 1.** Fermentation profile for avermectin B1b production

The results revealed that the proposed models based upon the Logistic and Piret Equations were significantly describing the relation between growth of *Streptomyces avermitilis* 41445 UV 45(m) 3, avermectin B1b production and substrate consumption. The kinetic parameter values for present fermentation in SM2 medium are represented in **Table 1**. The maximum specific growth rate ($\mu_{max}$) was 0.15 $h^{-1}$ in SM2 medium with 3.824 non-growth associated avermectin B1b production coefficients (β). The higher value of non-growth associated avermectin B1b production coefficient (β) than growth associated avermectin B1b production coefficient (α) revealed the process to be non-growth associated.

**Table 1.** Avermectin B1b fermentation by *S. avermitilis* 41445 UV 45(m) 3 in SM2 medium

| Parameters | Soluble Corn Starch |
|---|---|
| Maximum cell conc. $X_{max}$ ($g.L^{-1}$) | 25.6 |
| Initial cell conc. $X_0$ ($g.L^{-1}$) | 0.5 |
| Maximum specific growth rate $\mu_{max}$ ($h^{-1}$) | 0.1541 |
| Initial avermectin B1b Production $P_0$ ($g.L^{-1}$) | 0 |
| Maxi. avermectin B1b Production $P_{max}$ ($g.L^{-1}$) | 0.2586106 |
| Maintenance Coefficient $m_s$ | 0.003 |
| Avermectin B1b yield $(Y)_{P/S}$ | 0.737 |
| Growth associated avermectin B1b production coefficient, α | 0.001 |
| Non-Growth associated avermectin B1b production coefficient, β | 3.824 |
| Cell yield $Y_{X/S}$ | 0.644 |

*4.1. Effect of Different Carbon Sources*

Effect of different carbon sources on *S. avermitilis* 41445 UV 45(m) 3 growth and avermectin B1b production has been shown in **Table 2**. The kinetic parameter values were calculated from data obtained during fermentation process in SM2 media with each of the carbon sources. Results revealed that production and growth was effected significantly by type of carbon source used. Maximum specific growth rate ($\mu_{max}$) (1.29 $h^{-1}$) was obtained in medium having potato starch as carbon source followed by glucose (0.8 $h^{-1}$), maltose (0.34 $h^{-1}$), wheat starch (0.24 $h^{-1}$), lactose (0.16 $h^{-1}$) and soluble corn starch (0.15 $h^{-1}$). The production of secondary metabolites is related to cell biomass. In present work, maximum avermectin B1b production (420.02 $mg.L^{-1}$) was observed in medium with potato starch as carbon source having highest value of 31.74 g/L for $X_{max}$ ($g.L^{-1}$) followed by wheat starch, soluble corn starch, maltose, lactose and glucose. Rate of utilization of different carbon substrates and their effect on cell biomass and avermectin B1b production are shown in **Figures 1-4**.

**Table 2.** Kinetic parameter values of avermectin B1b fermentation by *S. avermitilis* 41445 UV 45(m) 3 using different types of carbon sources

| Parameters | Glucose | Maltose | Lactose | Soluble corn Starch | Wheat Starch | Potato Starch |
|---|---|---|---|---|---|---|
| Maximum cell conc. $X_{max}$ ($g.L^{-1}$) | 14.74 | 20.74 | 16.7 | 25.6 | 26.74 | 31.74 |
| Initial cell conc. $X_0$ ($g.L^{-1}$) | 0.1 | 0.1 | 0.2 | 0.5 | 0.3 | 0.5 |
| Maximum specific growth rate $\mu_{max}$ ($h^{-1}$) | 0.875 | 0.3458 | 0.16 | 0.1541 | 0.2416 | 1.291 |
| Initial avermectin B1b Production $P_0$ ($g.L^{-1}$) | 0 | 0 | 0 | 0 | 0 | 0 |
| Maxi. avermectin B1b Production $P_{max}$ (mg/L) | 130.01 | 143.11 | 138.01 | 182.14 | 258.61 | 420.02 |
| Maintenance Coefficient $m_s$ | 0.027 | 0.006 | 0 | 0.003 | 0.001 | 0.005 |
| Avermectin yield ($Y_{P/S}$) | 1.055 | 0.778 | 0.29 | 0.737 | 0.779 | 0.662 |
| Non-Growth associated avermectin B1b production coefficient, β | 11.36 | 3.124 | 3.72 | 3.824 | 56.66 | 22.29 |
| Growth associated avermectin B1b production coefficient, α | 0 | 0 | 0 | 0 | 0 | 0 |
| Cell yield $Y_{X/S}$ | 0.375 | 0.529 | 0.42 | 0.644 | 0.7344 | 1.077 |

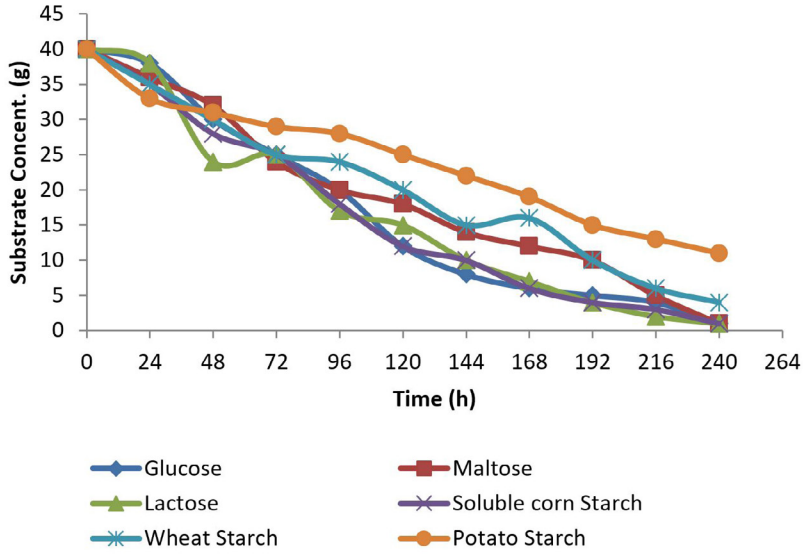


**Figure 2.** Rate of utilization of carbon substrates during fermentation

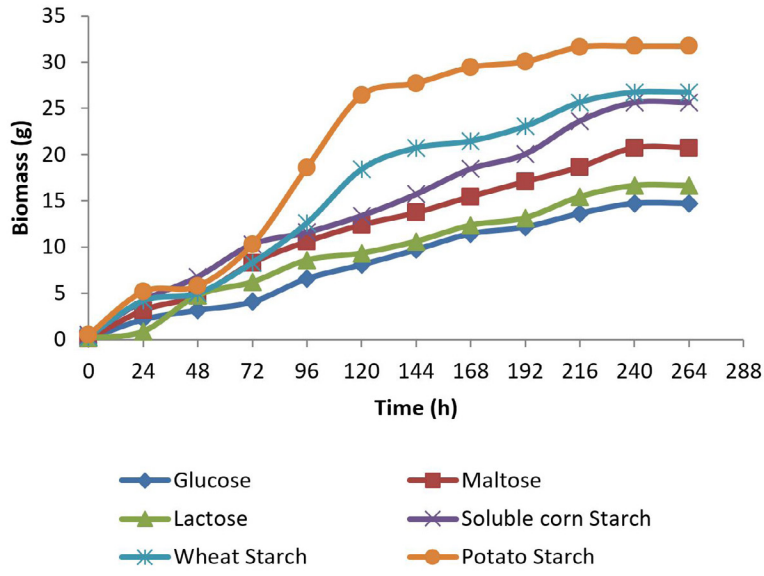


**Figure 3.** Effect of various carbon sources on cell biomass production

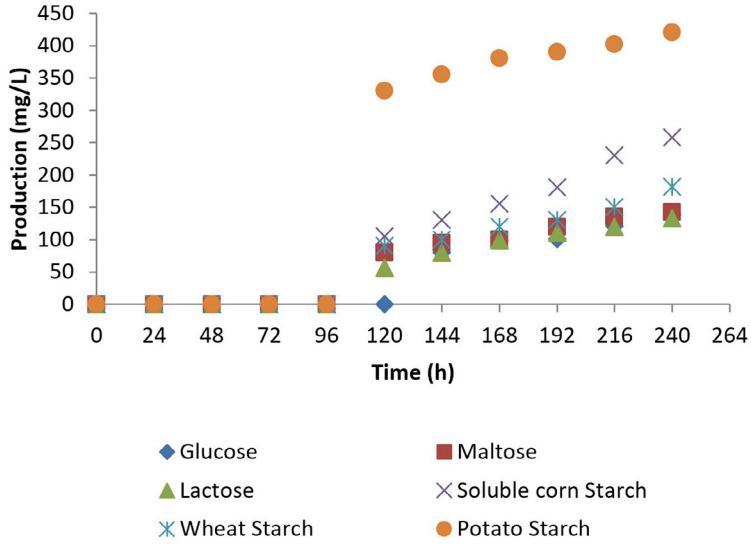


**Figure 4.** Effect of various carbon sources on avermectin B1b production

*4.2. Effect of pH*

Effect of different initial pH values on *S. avermitilis* 41445 UV 45(m) 3 growth and avermectin B1b production in medium with potato starch as carbon source has been shown in **Table 3**. The highest cell concentration ($X_{max}$) (21.3±0.04g.$L^{-1}$) was obtained at pH 7.5 with 180.04±0.03mg.$L^{-1}$ avermectin B1b production. Slight reduction in growth and avermectin B1b production were observed as the pH decreased from 7.5 to 7.0 with great inhibition at pH 6.5 followed by 6.0. The final cell concentration was about three times lowered at pH 6.0 as compared to the pH 7.5. However, two-time reduction in avermectin B1b production was observed at pH 6.0 as compared to the pH 7.5. As for as the cell yield ($Y_{X/S}$) and avermectin B1b yield ($Y_{P/S}$) is concerned, the cell yield vary significantly as the pH was reduced from 7.5 to 6.0. The results revealed that for higher avermectin B1b production and growth of *Streptomyces avermitilis* 41445 UV 45(m) 3, the pH of 7.5 is very suitable.

*4.3. Effect of Agitation Speed Streptomyces avermitilis 41445 UV 45(m) 3 Growth and Avermectin B1b Production in Medium with Potato Starch as Carbon Source at pH 7.5*

During shake flask fermentation, the effect of agitation speed on *S. avermitilis* 41445 UV 45(m) 3 growth and avermectin B1b production in potato starch medium

(pH 7.5) is shown in **Table 4**. The concentration of cell biomass did not vary considerably with changing the agitation speed however a slight increase in avermectin B1b production was observed with increasing the speed. The resulted revealed that highest production of avermectin B1b was obtained at agitation speed of 250 rpm. The other kinetic parameters such as ($Y_{X/S}$), ($Y_{P/S}$) and $\mu_{max}$ did not vary with speed of agitation.

**Table 3.** Kinetic parameter values of avermectin B1b fermentation by *S. avermitilis* 41445 UV 45(m) 3 using potato starch at different initial culture pH.

| Parameters | 6.0 | 6.5 | 7.0 | 7.5 |
|---|---|---|---|---|
| Maximum cell conc. $X_{max}$ ($g.L^{-1}$) | 7.01 | 10.12 | 18.4 | 21.3 |
| Initial cell conc. $X_0$ ($g.L^{-1}$) | 0.11 | 0.13 | 0.21 | 0.4 |
| Maximum specific growth rate $\mu_{max}$ ($h^{-1}$) | 0.11 | 0.12 | 0.10 | 0.16 |
| Initial avermectin B1b Production $P_0$ ($g.L^{-1}$) | 0 | 0 | 0 | 0 |
| Maxi. avermectin B1b Production $P_{max}$ ($mg.L^{-1}$) | 90.1 | 125.10 | 160.2 | 180.04 |
| Maintenance Coefficient $m_s$ | 0.001 | 0.003 | 0.003 | 0.004 |
| Avermectin B1b yield $Y_{X/S}$ | 0.412 | 0.47 | 0.50 | 0.889 |
| Non-Growth associated avermectin B1b production coefficient, β | 3.36 | 3.124 | 2.70 | 3.5 |
| Growth associated avermectin B1b production coefficient, α | 0 | 0 | 0 | 0 |

**Table 4.** Kinetic parameter values of avermectin B1b fermentation by *Streptomyces avermitilis* 41445 UV 45(m) 3 at medium pH 7.5 with variable agitation speed

| Parameters | 150.0 | 200.0 | 250.0 |
|---|---|---|---|
| Maximum cell conc. $X_{max}$ ($g.L^{-1}$) | 21.32 | 21.31 | 21.30 |
| Initial cell conc. $X_0$ ($g.L^{-1}$) | 0.11 | 0.13 | 0.4 |
| Maximum specific growth rate $\mu_{max}$ ($h^{-1}$) | 0.14 | 0.15 | 0.16 |
| Initial avermectin B1b Production $P_0$ ($g.L^{-1}$) | 0 | 0 | 0 |
| Maxi. avermectin B1b Production $P_{max}$ ($mg.L^{-1}$) | 175.02 | 178.03 | 180.04 |
| Maintenance Coefficient $m_s$ | 0.004 | 0.004 | 0.004 |
| Avermectin B1b yield $Y_{X/S}$ | 0.888 | 0.888 | 0.889 |
| Non-Growth associated avermectin B1b production coefficient, β | 3.4 | 3.3 | 3.5 |
| Growth associated avermectin B1b production coefficient, α | 0 | 0 | 0 |

## 5. Discussion

Fermentation is very complex process and it is not possible to depict all the process going on in a fermentation process. Production of secondary metabolites from microorganisms usually occurs after the cells entered the exponential growth phase from lag phase. The logistic rate equations have been used as alternative empirical equations to examine large data obtained during fermentations (5). In the present study, the avermectin B1b production also followed the typical trophophase-idiophase fermentation. Production of intracellular compounds is effected strongly by medium composition and the culture conditions (11). In the present study effect of various carbon sources and cultural condition was seen on avermectin B1b production and growth of *Streptomyces avermitilis* 41445 UV 45(m) 3 during submerged fermentation. Using the chemically defined media helped in better understanding of nutrient requirement for growth and secondary metabolite production as compared to the other complex medium (12). The results of the present study revealed that carbon source used as substrate along with initial culture pH and agitation speed played a key role for growth of *S. avermitilis* 41445 UV 45(m) 3 and avermectin B1b production. The modeling study revealed that the avermectin B1b production is non-

growth associated and occurred in stationary phase. During stationary phase, with increase in cell biomass, the production of avermectin B1b also increased gradually. Specific growth rate of 1.29 $h^{-1}$ gave higher cell yield (1.07 $g.g^{-1}$) and avermectin B1b yield (0.66 $mg.g^{-1}$) in SM2 medium having potato starch as carbon source. In a previous study it is reported that at specific growth rate of 0.36 $h^{-1}$, the maximum cell yield obtained was 0.37 $g.g^{-1}$ with xylanase yield being 154.09 $U.mg^{-1}$ from *E.coli* DH5α revealing that cultivation of cells at lower growth rate allowed the allocation of more cellular resources for product expression genes (11).

The medium and culture condition optimization for enhancing secondary metabolite production can be made more fruitful if the positive or negative effects of the components of medium and fermentation condition are known, as is explained during the xylanase production from *E.coli* (10,13,14). In the present study various carbon sources employed were glucose, lactose, maltose, soluble corn starch, wheat starch and potato starch. Maximum specific growth rate obtained was 1.2 $h^{-1}$ when the carbon source was the potato starch with maximum cell biomass 31.74±0.01 $g.L^{-1}$. A specific growth rate 0.06±0.007 $h^{-1}$ has been reported during the tylosin production from *Streptomyces fradiae* NRRL-2702 (15). Nitrogen source $NH_4Cl$ is associated with specific growth rate 0.69 $h^{-1}$ during xylanase production from *E.coli* DH5α (11). According to the previous works, maximum cell growth and total avermectin production (4.6 $mg.g^{-1}$) was obtained using sorghum seeds as solid state substrate (16). The highest titre of antimicrobial compound was obtained when maltose and yeast extract were used as carbon and nitrogen sources during the fermentation of *Streptomyces* sp. JRG-04 (17).

Production of enzymes and secondary metabolites from microorganisms at variable initial pH make them very selective towards a specific bioprocess as is reported by Naveena et al. (2012) (18). High specific growth rate and specific production rate were obtained at pH 7.0 from both the wild type and the mutant strain of *Streptomyces venezuelae* (17). In the present research work the highest specific growth rate obtained at pH 7.5 was 0.16 $h^{-1}$ from *Streptomyces avermitilis* 41445 UV 45(m) 3 with maximum specific production rate 180.04 $mg.L^{-1}$. This pH is associated with highest cell biomass 21.3±0.01 $g.L^{-1}$. It was reported by James et al. in "(1991) (19) that antibiotic production from *S. therrnoviolaceus* is highly dependent on medium pH and temperature. Maximum cell biomass was obtained at pH 5.5-6.5 at maximum growth rate of 0.15 $h^{-1}$ after which it began to decrease and so the Granaticin production. Farliahati et al. (2009) (11) reported 2122.5 $U.mL^{-1}$ and 4.59 $g.L^{-1}$ maximum xylanase production and maximum cell concentration respectively from *E. coli* DH5α at initial medium pH of 7.4. At this initial medium pH the maximum specific growth rate ($\mu_{max}$), growth associated xylanase production coefficient (α) and non-growth associated xylanase production coefficient (β) were 0.41 $h^{-1}$, 474.26 U $mg.cell^{-1}$ and 0 $U.mg.cell^{-1}.h^{-1}$, respectively. Reduction of medium pH resulted in lowered xylanase production (11). A pH of 7.5 at 28°C gave the maximum production of avermectin, antimicrobial compound, from *Streptomyces* sp. JRG-04 (17).

In previous research conducted by Farliahati et al. (2009) (11), it is reported that agitation speed does not contribute greatly towards the growth of microorganism as well as the enzyme production. Production of cell biomass varied a little with agitation speed. The optimal agitation speed reported by them is the 200 rpm with 2122.5 $U.mL^{-1}$ xylanase production (11). In the present research work the results are in close agreement with the results obtained previously. Maximum cell biomass of 21.32 $g.L^{-1}$ was obtained at agitation speed of 150 rpm. However specific rate and avermectin B1b production were maximum at agitation speed of 250 rpm, although not very different from that obtained at 150rpm.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Suggestion and Limitation:

The research work can also be done using the same bacterial strain by Solid state fermentation as there is possibility of achieving enhanced production of avermectin B1b as compared to submerged fermentation. However, SSF can take longer time period.

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