# KINETICS OF AVERMECTIN B1b PRODUCTION FROM MUTANT STRAIN OF STREPTOMYVES AVERMITILIS DSM 41445 IN SHAKE FLASK CULTURE

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***ABSTRACT:*** *Kinetics of intracellular avermectin B1b production was studied in shake flask culture during submerged fermentation. Mathematical models based upon Logistic and Luedeking-Piret Equations have been used to investigate the kinetics of avermectin B1b production and substrate utilization from Streptomyces avermitilis 41445 UV 45(m) 3. Effects of various carbon sources, pH and agitation speed on microbial growth and product formation were evaluated. Maximum avermectin B1b production (420.02 ±0.01 mg/L) and cell biomass (31.74 ±0.05 g/L) were obtained in media having potato starch as carbon substrate, medium pH of 7.5 with agitation speed of 250 rpm. Maximum specific growth rate ($\mu_{max}$), growth associated avermectin B1b production coefficient (α) and non-growth associated avermectin B1b production coefficient (β) obtained were 0.16$h^{-1}$, 0 mg $cell^{-1}$ $h^{-1}$ and 3.5 mg $cell^{-1}$ $h^{-1}$ respectively. From above results we concluded that avermectin B1b production was non-growth associated process.*

**Key words:** Streptomyces avermitilis 41445 UV 45(m) 3; submerged fermentation; Logistic and Luedeking-Piret Equations

## INTRODUCTION

*Streptomyces avermitilis* belongs to gram positive, aerobic and mesophilic *Actinomycetes*. It is characteristically differentiated into branched substrate mycelium and aerial hyphae which are distinguished into long, compact spiral chains. 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 avermectins [2]. Avermectins lacking antimicrobial activities strongly exhibit broad spectrum potent anthelmintic and insecticidal properties [3]. Large scale production of drugs and drug precursors by microbial fermentation is of utmost importance for pharmaceutical industry [4]. Owing to complex and diverse chemical structure of these secondary metabolites, their production in large quantities under normal conditions is not possible [5]. Microbial production of secondary metabolites can be enhanced through several strain improvement strategies [6]. Strain improvement through mutagenesis has resulted in about hundred times more secondary metabolites during fermentation as compared to the wild strain [7]. The most convenient and safe method for mutagenesis is UV irradiation [8,9]. In the present research work, the mutant strain of *Streptomyces avermitilis* DSM 41445 obtained through UV Irradiation has been used for the kinetic parameter study.

Substrate used in the cultivation medium not only significantly effects the production of avermectins from the *Streptomyces avermitilis* but also the synthesis of certain enzymes, used in sugar metabolism [10]. Microbial production of antibiotics is of great economical importance because industrial fermentations are moving from traditional approaches towards the simpler and controlled processes. Kinetic models enable the engineers to construct and control microbial process in an elegant way [11]. Structured and unstructured models taking into account the mathematical and experimental designs are commonly used for expression of microbial processes [12]. Batch experiments were performed for kinetic parameters evaluation and operation conditions optimization as reported previously [13]. Initial substrate concentration ($S_0$) to initial cell biomass concentration ($X_0$) ratio strongly influenced the biomass yield [14]. Most studied biokinetic coefficients include specific growth rate (μ), maximum rate of substrate utilization per unit mass of microorganism (k), half velocity constant ($K_S$) and maximum cell yield (Y) [15,16].

Due to the complex nature of *Streptomyces avermitilis*, the kinetics of intracellular avermectin production has been studied improperly [17,18]. Avermectin B1b is the minor component of commercially available abamectin (mixture of B1a & B1b) and has been used as anthelmintic and insecticidal agent [19]. In the present study, the shake flask fermentation has been done to investigate the kinetic parameters for *Streptomyces avermitilis* 41445 UV 45(m) 3 growth, avermectin B1b production and substrate utilization using Logistic and Luedeking-Piret Equations.

## EXPERIMENTAL

### Microorganism and Culture Maintenance

*Streptomyces avermitilis* 41445 UV 45(m) 3 obtained through UV mutagenesis (Siddique et al., 2013b) was used in the present study. The spores of *Streptomyces avermitilis* DSM 41445 were exposed to UV rays in dark room at a distance of 10cm for 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 minutes from UV lamp (DESAGA, Sarstedt-Gruppe, MinUVIS UV Lamp) (λ=320 nm). The spores were spread nutrient agar plates and were incubated at 28°C for 24 hours [20] (Khattab & Abdel-Aziz, 2012). The lethality rate, mutation rate and positive mutation rate were calculated using the following equations described previously [21].

Lethality rate = (T/U) × 100%

Mutation rate = (M/T) × 100%

Positive mutation rate = (P/M) × 100%

Here U = No. of colonies without UV treatment

T = No. of colonies after UV treatment

M = total CFU of mutant strain

P = CFU of the mutants with avermectin B1b production more than that of the parent *Streptomyces avermitilis* DSM 41445.

**Inoculum Preparation**

Culture from the nutrient agar slants was inoculated into DSMZ specified YMG medium (Yeast extract malt extract glucose medium) consisted of (g/L in distilled water) glucose 4.0 g, yeast extract 4.0 g, malt extract 10.0 g and $CaCO_3$ 2.0 g used as seed medium. The pH of medium was adjusted at 6.5 before sterilization. After sterilization at 121°C for 15 minutes, the medium pH was finalized at 7.0±2 with the addition of appropriate amount of $CaCO_3$ [3,21]. The medium was incubated at 31°C in the water bath shaker (Eyela, Japan) for 24 hours at 150 rpm.

**Fermentation Medium**

The production of avermectin B1b from *Streptomyces avermitilis* 41445 UV 45(m) 3 was studied in fermentation medium SM2. 10% v/v inoculum was inoculated in fermentation medium and incubated at 31°C in water bath shaker for 10 days at 150 rpm. The fermentation medium consisted of (g/L) soluble corn starch 50.0 g, KCl 0.1 g, NaCl 0.5 g, yeast extract 2.0 g, $MgSO_4.7H_2O$ 0.1 g, $CaCO_3$ 0.8 g and α-amylase 0.1 g. 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 *Streptomyces avermitilis* 41445 UV 45(m) 3 and avermectin 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 6.0-7.5 on growth of *Streptomyces avermitilis* 41445 UV 45(m) 3 and avermectin B1b production. The effect of agitation speed was investigated at initial culture pH of 7.5. All the experiments were performed in triplicates.

**2.4 Analytical Procedure**

Samples taken at 12 hours intervals from fermentation medium were centrifuged (H-1500FR Japan) at 8000 rpm for 15 minutes at 4°C. Cell growth and avermectin B1b production were observed after cell biomass analysis. The cell pellet suspended in normal saline was subjected to spectrophotometer for optical density 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 hours in oven.

For extraction of intracellular avermectin B1b from fermentation broth, cell biomass was taken and supernatant was discarded. Cell biomass in form of pallet was mixed with appropriate amount of methanol in pestle and motor and crushed to completely dissolve it. The mixture was then centrifuged for the separation of cell biomass and the supernatant was collected for avermectin B1b analysis 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. Individual components were eluted by methanol:acetonitrile (98 : 2 v/v) at a flow rate of 0.5 ml/min with a UV absorbance at 246 nm [22].

**Kinetics and Mathematical Models**

The growth of *Streptomyces avermitilis* 41445 UV 45(m) 3 and production of avermectin B1b were calculated using logistic and Luedeking-Piret equations respectively [23] and are given below.

**Microbial Growth**

The Logistic equation can be describes as follow:

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

**Substrate Consumption**

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

**Avermectin B1b production**

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

Where

X = the concentration of cell biomass (g/L)

$X_{max}$ = the maximum concentration of cell biomass (g/L)

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

S = Substrate concentration (g/L)

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

$m_s$ = maintenance coefficient (g/g/h)

α = growth associated avermectin B1b production coefficient (mg/g)

β = non growth associated avermectin B1b production coefficient (mg/g/h)

t = time of fermentation (h)

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

**RESULTS AND DISCUSSION**

Comparison of *Streptomyces avermitilis* 41445 UV 45(m) 3 growth, avermectin B1b production and substrate consumption in SM2 medium during submerged fermentation is shown in Fig 1. The synthetic medium 2 (SM2) has been used previously [22]. From Figure 1, it is clear that growth arrived at stationary phase after 72 hours in SM2 medium with 10.30±0.02 g/L cell biomass. Avermectin B1b production was not observed up to 72 hours of fermentation and was started in stationary phase revealing the process to be non growth associated. After the growth entered the stationary phase, the production of avermectin B1b increased with increasing the cell biomass. The production of avermectin B1b was maximum (258.6±0.01) at $10^{th}$ day of fermentation after that it began to decrease. Substrate which is soluble corn starch in the present case was consumed for microbial growth and avermectin B1b production and completely utilized at end of fermentation process.

Fermentation is very complex process and it is not possible to depict all the processes going on [11,24]. Production of secondary metabolites from microorganisms usually occurs after the cells entered the exponential growth phase from lag phase [25]. The logistic rate equations have been used as alternative empirical equations to examine the fermentation data [11]. In the present study, avermectin B1b production also followed typical trophophase-idiophase fermentation

pattern. it is reported that antibiotic production from microorganism during fermentation process normally follows the trophophase-idiophase fermentation pattern [11]. Production of intracellular compounds is effected strongly by medium composition and the culture conditions [26]. In the present study effects of various carbon sources and cultural condition were 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 [26] as compared to the other complex medium. 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 during microbial growth and product formation. The modeling study revealed that avermectin B1b production is non growth associated and occurred in stationary phase. During stationary phase, avermectin B1b production and concentration of cell biomass gradually increased with time. 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 where potato starch has been used as carbon source. In a study conducted previously,

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

| Parameters | Soluble corn Starch |
|---|---|
| Maximum cell conc. $X_{max}$ (g/L) | 25.6 |
| Initial cell conc. $X_0$ (g/L) | 0.5 |
| Maximum specific growth rate μ | 0.1541 |
| Initial avermectin B1b Production | 0 |
| Maxi. avermectin B1b Production | 0.2586106 |
| Maintenance Coefficient $m_s$ | 0.003 |
| $Y_{P/S}$ | 0.737 |
| Growth associated avermectin B1b | 0.001 |
| Non-Growth associated avermectin | 3.824 |
| Cell yield $Y_{X/S}$ | 0.644 |

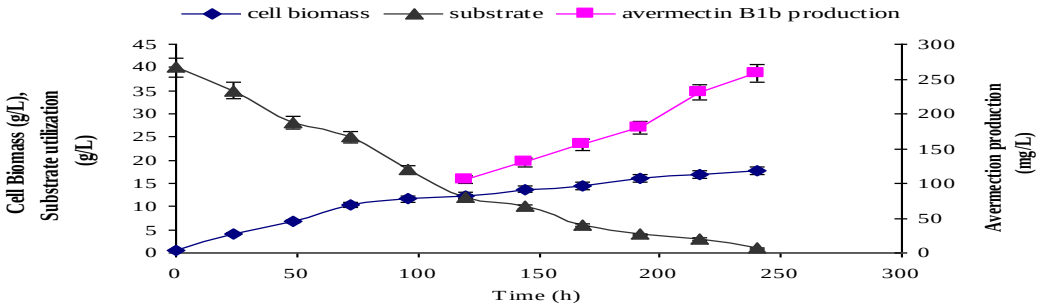


**Fig. 1 Fermentation Profile for Avermectin B1b Production**

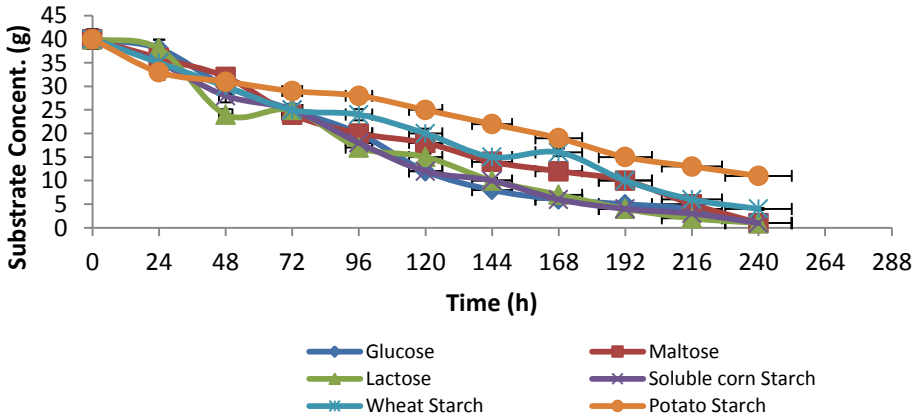


**Fig. 2. Rate of Carbon Substrate Utilization during Fermentation**

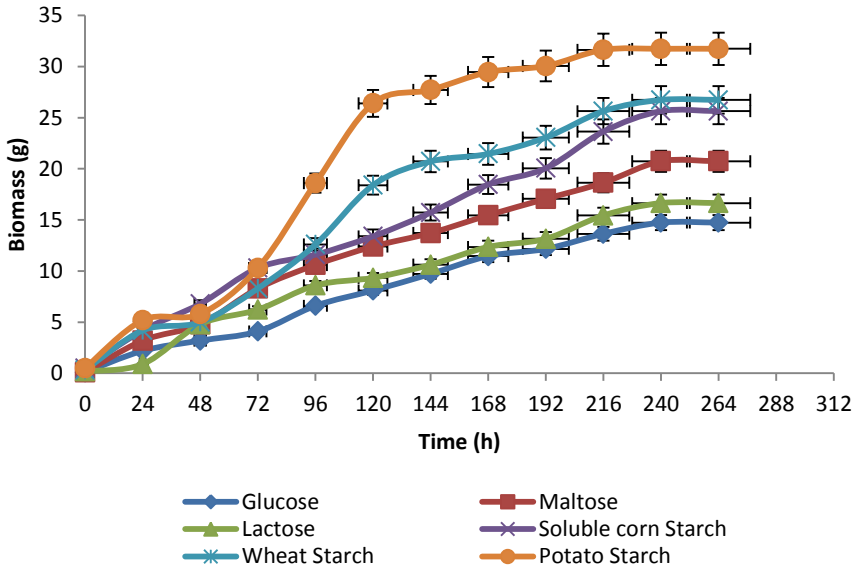


**Fig. 3. Effect of Various Carbon Sources on Cell Biomass Production**

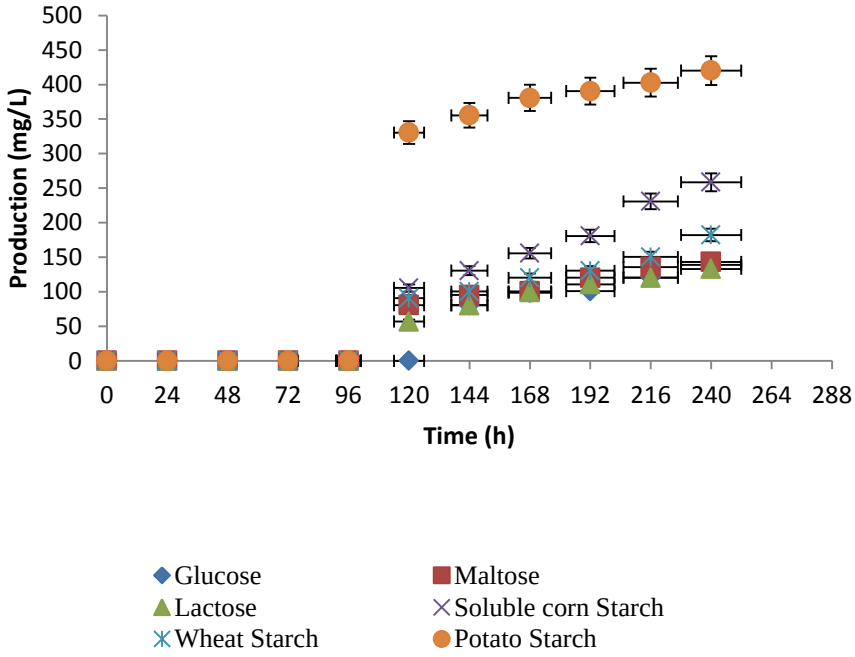


**Fig. 4. Effect of Various Carbon Sources on Avermectin B1b Production**

**Table 2. Kinetic Parameter Values of Avermectin B1b Fermentation by *Streptomyces avermitilis* 41445 UV 45(m) 3 using Different Carbon Sources**

| Parameters | Carbon Sources | | | | | |
|---|---|---|---|---|---|---|
| | Glucose | Maltose | Lactose | Soluble [illegible] | Wheat [illegible] | Potato Starch |
| $X_{max}$ (g/L) | 14.74 | 20.74 | 16.7 | 25.6 | 26.74 | 31.74 |
| $X_0$ (g/L) | 0.1 | 0.1 | 0.2 | 0.5 | 0.3 | 0.5 |
| $\mu_{max}$ ($h^{-1}$) | 0.875 | 0.3458 | 0.16 | 0.1541 | 0.2416 | 1.291 |
| $P_0$ (g/L) | 0 | 0 | 0 | 0 | 0 | 0 |
| $P_{max}$ (mg/L) | 130.01 | 143.11 | 138.01 | 182.14 | 258.61 | 420.02 |
| $m_s$ | 0.027 | 0.006 | 0 | 0.003 | 0.001 | 0.005 |
| $Y_{P/S}$ | 1.055 | 0.778 | 0.29 | 0.737 | 0.779 | 0.662 |
| B | 11.36 | 3.124 | 3.72 | 3.824 | 56.66 | 22.29 |
| A | 0 | 0 | 0 | 0 | 0 | 0 |
| $Y_{X/S}$ | 0.375 | 0.529 | 0.42 | 0.644 | 0.7344 | 1.077 |

Note: $X_{max}$= Maximum cell biomass produced in grams per liter, $X_0$= initial cell biomass, $\mu_{max}$= maximum specific growth rate per hour, $P_0$= initial product formation, $P_{max}$=maximum product formation, ms= maintenance Coefficient, $Y_{P/S}$= product yield, β= Non-Growth associated avermectin B1b production coefficient, α= Growth associated avermectin B1b production coefficient, $Y_{X/S}$= Cell biomass yield over substrate consumed.

**Table 3. Kinetic Parameter Values of Avermectin B1b Fermentation by *Streptomyces avermitilis* 41445 UV 45(m) $^3$ using Potato Starch at Different Initial Culture pH**

| Parameters | pH | | | |
|---|---|---|---|---|
| | 6.0 | 6.5 | 7.0 | 7.5 |
| $X_{max}$ (g/L) | 7.01 | 10.12 | 18.4 | 21.3 |
| $X_0$ (g/L) | 0.11 | 0.13 | 0.21 | 0.4 |
| $\mu_{max}$ ($h^{-1}$) | 0.11 | 0.12 | 0.10 | 0.16 |
| $P_0$ (g/L) | 0 | 0 | 0 | 0 |
| $P_{max}$ (mg/L) | 90.1 | 125.10 | 160.2 | 180.04 |
| $m_s$ | 0.001 | 0.003 | 0.003 | 0.004 |
| $Y_{p/s}$ | 0.1 | 0.5 | 0.4 | 0.2 |
| B | 3.36 | 3.124 | 2.70 | 3.5 |
| α | 0 | 0 | 0 | 0 |
| $Y_{X/S}$ | 0.412 | 0.47 | 0.50 | 0.889 |

**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 | Agitation speed (rpm) | | |
|---|---|---|---|
| | 150.0 | 200.0 | 250.0 |
| $X_{max}$ (g/L) | 21.32 | 21.31 | 21.30 |
| $X_0$ (g/L) | 0.11 | 0.13 | 0.4 |
| $\mu_{max}$ ($h^{-1}$) | 0.14 | 0.15 | 0.16 |
| $P_0$ (g/L) | 0 | 0 | 0 |
| $P_{max}$ (mg/L) | 175.02 | 178.03 | 180.04 |
| $m_s$ | 0.004 | 0.004 | 0.004 |
| $Y_{p/s}$ | 0.1 | 0.7 | 0.5 |
| B | 3.4 | 3.3 | 3.5 |
| α | 0 | 0 | 0 |
| $Y_{X/S}$ | 0.888 | 0.888 | 0.889 |

a specific growth rate of 0.048 $h^{-1}$ has been observed for *Streptomyces coelicolor* during fermentation process [27]. Antibiotic biosynthesis from *Streptomycetes* is normally restricted to non growing stationary phase biomass. This is due to the presence of a variety of repressors of secondary metabolites production such as carbon source, level of ammonia or production of antibiotic pathway enzymes [28]. In a previous study it is reported that at te specific growth rate of 0.36 $h^{-1}$, the maximum cell yield obtained was 0.37 $g.g^{-1}$ with 154.09 U $mg^{-1}$ xylanase production from *E.coli* DH5α. The results revealed that cultivation of cells at lower growth rate allowed the allocation of more cellular resources for product expression genes [26].

The results of t present study revealed that the proposed models based upon the Logistic and Luedeking-Piret equations significantly described the relation between growth of *Streptomyces avermitilis* 41445 UV 45(m) 3, avermectin B1b production and substrate utilization. The kinetic parameter values in SM2 medium are listed in Table 1.

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. In a previous study, it is reported that synthesis of dipicolinic acid (DPA) from *Penicillium citreoviride* showed typical non growth associated kinetics of secondary metabolites [29]. Synthesis of DPA resumed during idiophase and continued through stationary phase of growth. In previous study it is reported that primary metabolites are produced during active growth phase of microorganism and show growth associated kinetics. In contrast, the secondary metabolites are the compounds produced after active growth has taken place and are of no vital function for the microbe. Therefore, the secondary metabolites showed non growth associated kinetics in most of the circumstances [30].

**Effect of Different Carbon Sources on Streptomyces avermitilis 41445 UV 45(m) 3 Growth and Avermectin B1b Production**

Effects of different carbon sources on *Streptomyces avermitilis* 41445 UV 45(m) 3 growth and avermectin B1b production are listed in Table 2. Kinetic parameter values were calculated from data obtained during fermentation process in SM2 medium with changed carbon source. Results revealed that production and growth were significantly affected by nature 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 $h^{-1}$). Production of secondary metabolites is related to cell biomass. In present research work maximum avermectin B1b production (420.02±0.01 mg/L) was observed in medium with potato starch as carbon source having highest $X_{max}$ (g/L) value of 31.74 g/L followed by wheat starch, soluble corn starch, maltose, lactose and glucose. Rate of utilization of different carbon substrate and their effects on cell biomass production and product formation are shown in Figures 2, 3 and 4 respectively.

The medium and culture condition optimization for enhanced secondary metabolite production can be made more fruitful if positive or negative effects of medium components and fermentation condition are known [31,32,33]. In the present study various carbon source employed were glucose, lactose, maltose, soluble corn starch, wheat starch and potato starch. Maximum specific growth rate obtained was 1.2 $h^{-1}$ in presence of potato starch with maximum cell biomass 31.74±0.01 g/L. Specific growth rate 0.06±0.01 $h^{-1}$ has been reported during the tylosin production from *Streptomyces fradiae* NRRL-2702 [34]. Nitrogen source $NH_4Cl$ is associated with specific growth rate 0.69 $h^{-1}$ during xylanase production from *E.coli* DH5α [26].

**Effect of pH on Streptomyces avermitilis 41445 UV 45(m) 3 Growth and Avermectin B1b Production in Medium with Potato Starch as Carbon Source**

Effects of different initial pH on *Streptomyces avermitilis* 41445 UV 45(m) 3 growth and avermectin B1b production are listed in Table 3. The highest cell concentration ($X_{max}$) (21.3±0.04 g/L) was obtained at pH 7.5 with 180.04±0.03 mg/L avermectin B1b production. Slight reduction in growth and avermectin B1b production observed as 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 pH 7.5. As for as the cell yield ($Y_{X/S}$) and avermectin B1b yield ($Y_{P/S}$) are concerned, the cell yield vary significantly as pH was reduced from 7.5 to 6.0. The results revealed that for higher product formation and microbial growth, the medium with pH 7.5 is suitable.

Production of enzymes and secondary metabolites from microorganisms at variable initial pH make them very selective towards a specific bioprocess as reported previously [13]. 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*. In the present study, 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. This pH is associated with highest cell biomass 21.3±0.01 g/L. It is reported that antibiotic production from *S. therrnoviolaceus* was 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 [28]. In a previous research it is reported that 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 0.0 U $mgcell^{-1}h^{-1}$ respectively. Reduction of medium pH resulted in lowered xylanase production [26].

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

During shake flask fermentation, effects of agitation speed on *Streptomyces avermitilis* 41445 UV 45(m) 3 growth and avermectin B1b production are listed in Table 4. The concentration of cell biomass did not vary considerably with agitation speed, however a slight increase in avermectin B1b production was observed. Results revealed that highest avermectin B1b was obtained at agitation speed of 250 rpm. Other kinetic parameters such as ($Y_{X/S}$), ($Y_{P/S}$) and $\mu_{max}$ did not vary with speed of agitation.

In a previous research, it was reported that agitation speed did not contribute greatly towards growth of microorganism as well as enzyme production. Production of cell biomass varied a little with agitation speed. The optimal agitation speed reported by them is the 200rpm with 2122.5 U/mL xylanase production [26]. In present research work, results are in close agreement with previous studies. Maximum cell biomass of 21.32±0.02 g/L was obtained at agitation speed of 150 rpm. However specific rate and avermectin B1b production were maximum at agitation speed 250 rpm although not very different from that obtained at 150 rpm.

## CONCLUSION

The present research is the first comprehensive kinetic study of avermectin B1b production from mutant strain of *Streptomyces avermitilis* 41445 UV 45(m) 3 in shake flask culture. The results of thepresent research revealed the process of avermectin B1b production to be non growth associated.

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