# Chapter 5

# Studies on most persistent and dominant denitrifying bacterium in continuously operated MBBR

## 5.1 Introduction

*Thauera* contributes to waste treatment by detoxifying targeted recalcitrant compounds, humic acids and metal ions and convert them into more easily utilizable compounds that serve nutrients for other microorganisms (Heider and Fuchs, 2015). Different isolates of genus *Thauera* from various environments have been found to be capable of denitrification, production of EPS components, biological mineralization and degradation of methylbenzene, phenol and linalool under aerobic or anaerobic conditions (Qiao et al., 2018). Its ability to degrade nitrate and other recalcitrant pollutants makes *Thauera* a preferred organism for bioremediation. In literature, *Thauera* spp. has been shown to play an important role in anaerobic/anoxic/oxic reactor (Zhao et al., 2018), in activated sludge system reactor (Liang et al., 2015) denitrifying biofilter (Cui et al., 2017), denitrifier coupled with aerobic denitrification (Ji et al., 2015), partial denitrification (Du et al., 2019), simultaneous nitrification and denitrification (Wang and He, 2020) and hydrogen-oxidizing autotrophic denitrification (Mao et al., 2013).

Results of whole metagenomics study discussed in chapter 4 revealed that dMBBR bioaugmented with consortium DC5 showed *Thauera* spp. as the most dominant and persistent organism in the developed biofilm after 300 days of continuous operation. Therefore, this chapter discusses further studies carried out with the isolated strain of denitrifying bacterium *Thauera* sp. V14 to understand its potential in denitrification reactors.

## 5.2 Materials and methods

### 5.2.1 Microorganism, media and growth condition

Pure culture of *Thauera* sp.V14 was preserved at 4-8 °C on the CPNA agar plate after 48 h of incubation at 37 °C (media composition as mentioned in chapter 2 section 2.2.1.2). Flask level and reactor level studies with *Thauera* sp.V14 were carried out in MM2 medium (media composition as mentioned in chapter 2 section 2.2.2.3).

### 5.2.2 Auto-aggregation ability of *Thauera* sp.V14

*Thauera* sp. V14 was grown in PNB for 18–24 h and centrifuged at 8000 rpm for 7 min. Harvested cells were washed twice with PBS and then resuspended in PBS with absorbance set at 0.6 (A0) $OD_{600nm}$. This suspension was inoculated in 50 ml of MM2 medium and allowed to stand for 8 h to allow aggregation and the $OD_{600}$nm was measured again (At). The auto-aggregation index was calculated by Eq. (1) to evaluate the auto-aggregation capacity of *Thauera* sp. V14 (Hong et al., 2020).

$$\text{Auto aggregation index (\%)} = \left(1-\frac{At}{A0}\right) \times 100 \quad (1)$$

Where $A_t$ and $A_0$ are $OD_{600nm}$ at time t=1, 2, 3, 4, 5, 6, 7 and 8 and at time t=0 time respectively.

### 5.2.3 Hydrophobicity of *Thauera* sp.V14

The hydrophobicity of cells was determined by measuring bacterial adhesion. *Thauera* sp.V14 was grown in PNB for 18–24 h and centrifuged at 8000 rpm for 7 min. Harvested cells were washed twice with PBS and resuspended in PBS with absorbance set at 0.3 and inoculated in 50 ml of MM2 medium. Aliquots were drawn at every 24 h intervals for 120 h. To check the hydrophobicity, 4 ml of cell suspension with 1 ml of hexadecane was vortexed for 2 min and the two phases were allowed to separate for 15 min. The aqueous phase was removed carefully, and its $OD_{546nm}$ was taken. The cell surface hydrophobicity was calculated using Eq. (2) (Hong et al., 2020).

$$\text{Hydrophobicity(\%)} = \left(1-\frac{OD546}{0.3}\right) \times 100 \quad (2)$$

### 5.2.4 Flask level denitrification efficiency of *Thauera* sp.V14

Denitrification studies were carried out with *Thauera* sp.V14 with an initial nitrate concentration of 765 mg $L^{-1}$ in 250 ml flask. It was grown in PNB for 18–24 h and centrifuged at 8000 rpm for 7 min. The cell pellet was washed twice with PBS and resuspended in PBS with absorbance set at 0.5 $OD_{600nm}$. 1 % of this prepared inoculum was inoculated in a flask containing 100 ml of MM2 medium and was incubated at 37 °C

for 48 h in static conditions; aliquots were drawn at 24 h intervals and assayed for nitrate, nitrite and ammonia according to APHA1998 (section 2.2.13).

Denitrification efficiency was calculated by the following formula

$$\text{Denitrification Efficiency (\%)} = \frac{[NO_3]\text{In} - [NO_3]\text{Out}}{[NO_3]\text{In}} \times 100$$

## 5.2.5 Effect of temperature on morphology and motility of *Thauera* sp.V14

PNB was inoculated with *Thauera* sp.V14 and incubated at 37 ºC for 24 h. 5 µl aliquot of overnight grown *Thauera* culture was placed on CPNA plates. The plates were sealed with Parafilm and incubated at different temperatures (25 ºC, 30 ºC, 37 ºC and 40 ºC) for 72 h.

For swarming motility test plates were prepared with soft Luria Agar (LA) with 0.8 % agar and 5 µl aliquots of overnight grown *Thauera* culture was placed in the center of the plates. The inoculated plates were then incubated at RT 30 ºC, 37 º C, and 40 ºC for 72 h to observe swarming.

## 5.2.6 Optimization of factors affecting *Thauera* sp. V14 biofilm and denitrification efficiency (One factor at a time (OFAT))

### 5.2.6.1 Effect of metal ions

Different concentration of $MgCl_2$ (0, 0.1, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 mM), $CaCl_2$, (0, 0.1, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 mM) were supplemented individually in MM2 media. 40 µl of *Thauera* sp.V14 was inoculated in 2 ml of MM2 media (supplemented with the different concentrations of the ions) and dispensed in microtiter plates. After 48 h of incubation, denitrification efficiency and biofilm forming ability of *Thauera* sp.V14 were checked.

### 5.2.6.2 Effect of inoculum size

Different concentration of inoculum 2.5, 4, 5, 6, 7, 8, 9 and 10 % was individually inoculated in 2 ml of MM2 media in microtiter plate. After 48 h of incubation, denitrification efficiency and biofilm forming ability of *Thauera* sp.V14 were checked.

**5.2.6.3 Effect of amyloid supplementation**

Different concentrations (0, 2, 4, 6, 8, 10, 20, 30, 40, 50, 60, 70, 80 and 90 mg/ml) of purified amyloid with 40 µl of *Thauera* sp.V14 was individually added in 2 ml of MM2 media. After 48 h of incubation, denitrification efficiency and biofilm forming ability of *Thauera* sp.V14 were checked.

**5.2.6.4 Effect of NaCl**

0.2 and 0.5 % of NaCl with 40 µl of *Thauera* sp.V14 was inoculated individually in 2 ml of MM2 media. After 48 h of incubation, denitrification efficiency and biofilm forming ability of *Thauera* sp.V14 were checked.

**5.2.6.5 Effect of DMSO**

0.05, 0.5, 1 and 1.5 % of DMSO and 40 µl of *Thauera* sp.V14 was individually inoculated in 2 ml of MM2 media. After 48 h of incubation, denitrification efficiency and biofilm forming ability of *Thauera* sp.V14 were checked.

**5.2.6.6 Effect of Ethanol**

0.05, 0.5, 1 and 1.5 % of ethanol and 40 µl of *Thauera* sp.V14 was individually inoculated in 2 ml of MM2 media. After 48 h of incubation, denitrification efficiency and biofilm forming ability of *Thauera* sp.V14 were checked.

**5.2.6.7 Plackett-Burman Design**

Several factors must be affecting biofilm and denitrification efficiency of *Thauera* sp.V14. To examine the role of each component the Plackett-Burman design method was used. Plackett-Burman design is one of the widely used statistical method for the assortment of different factors. The statistically significant variables were selected on the basis of F test. Independent variables namely, Peptone (A), Yeast extract (B), Purified amyloid (C), DMSO (D), Ethanol (E), NaCl (F), $MgCl_2$ (G) and $CaCl_2$ (H) were used for the investigation. Dummy 1(I), Dummy 2 (J) and Dummy 3(K) were kept to fit the design and reduce the experimental errors. The experimental ranges of each variable were kept from 0.5 % (Low) to 3 % (High) concentration. The experiment was designed using Design expert software version 13.0 of StatEase, USA.

*Thauera* sp. V14 was grown in PNB for 18–24 h and centrifuged at 8000 rpm for 7 min. Harvested cells were washed twice with PBS and then resuspended in PBS with absorbance set at 0.5 $OD_{600nm}$. Then 9 % of cell suspension was inoculated in the MM2 media and incubated at 37 ºC for 48 h and denitrification efficiency and biofilm forming ability of the *Thauera* sp.V14 were determined. Plackett-Burman design was performed in the microtiter plates where each factor was added as mentioned in the Table 5.1.

**Table 5.1 Plackett-Burman experimental design**

| Run | A | B | C | D | E | F | G | H | I | J | K |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.5 | 0.5 | 0.5 | 3 | 0.5 | 3 | 3 | 0.5 | 3 | 3 | 3 |
| 2 | 0.5 | 0.5 | 3 | 0.5 | 3 | 3 | 0.5 | 3 | 3 | 3 | 0.5 |
| 3 | 0.5 | 3 | 0.5 | 3 | 3 | 0.5 | 3 | 3 | 3 | 0.5 | 0.5 |
| 4 | 3 | 3 | 0.5 | 0.5 | 0.5 | 3 | 0.5 | 3 | 3 | 0.5 | 3 |
| 5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| 6 | 3 | 3 | 3 | 0.5 | 0.5 | 0.5 | 3 | 0.5 | 3 | 3 | 0.5 |
| 7 | 0.5 | 3 | 3 | 0.5 | 3 | 3 | 3 | 0.5 | 0.5 | 0.5 | 3 |
| 8 | 3 | 0.5 | 3 | 3 | 3 | 0.5 | 0.5 | 0.5 | 3 | 0.5 | 3 |
| 9 | 3 | 3 | 0.5 | 3 | 3 | 3 | 0.5 | 0.5 | 0.5 | 3 | 0.5 |
| 10 | 3 | 0.5 | 0.5 | 0.5 | 3 | 0.5 | 3 | 3 | 0.5 | 3 | 3 |
| 11 | 0.5 | 3 | 3 | 3 | 0.5 | 0.5 | 0.5 | 3 | 0.5 | 3 | 3 |
| 12 | 3 | 0.5 | 3 | 3 | 0.5 | 3 | 3 | 3 | 0.5 | 0.5 | 0.5 |

### 5.2.6.8 Response Surface Methodology (RSM)

The significant factors Ethanol, $CaCl_2$, Peptone, DMSO and Yeast extract were selected based on Plackett-Burman analysis. Using Design expert software (version 13) various concentration of factors were selected and response surface methodology (RSM) was performed in microtiter plates where each factor added as indicated in Table 5.2.

**Table 5.2 RSM experimental design**

| Std. | Run | Peptone | Yeast extract | DMSO | Ethanol | $CaCl_2$ |
|---|---|---|---|---|---|---|
| | | % | % | % | % | % |
| 48 | 1 | 1.75 | 1.75 | 1.75 | 1.75 | 1.75 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 17 | 2 | 0.5 | 0.5 | 0.5 | 0.5 | 3 |
| 12 | 3 | 3 | 3 | 0.5 | 3 | 0.5 |
| 4 | 4 | 3 | 3 | 0.5 | 0.5 | 0.5 |
| 40 | 5 | 1.75 | 1.75 | 1.75 | 4.72 | 1.75 |
| 25 | 6 | 0.5 | 0.5 | 0.5 | 3 | 3 |
| 23 | 7 | 0.5 | 3 | 3 | 0.5 | 3 |
| 20 | 8 | 3 | 3 | 0.5 | 0.5 | 3 |
| 33 | 9 | -1.22 | 1.75 | 1.75 | 1.75 | 1.75 |
| 35 | 10 | 1.75 | -1.22 | 1.75 | 1.75 | 1.75 |
| 3 | 11 | 0.5 | 3 | 0.5 | 0.5 | 0.5 |
| 41 | 12 | 1.75 | 1.75 | 1.75 | 1.75 | -1.22 |
| 47 | 13 | 1.75 | 1.75 | 1.75 | 1.75 | 1.75 |
| 28 | 14 | 3 | 3 | 0.5 | 3 | 3 |
| 22 | 15 | 3 | 0.5 | 3 | 0.5 | 3 |
| 7 | 16 | 0.5 | 3 | 3 | 0.5 | 0.5 |
| 29 | 17 | 0.5 | 0.5 | 3 | 3 | 3 |
| 1 | 18 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| 19 | 19 | 0.5 | 3 | 0.5 | 0.5 | 3 |
| 11 | 20 | 0.5 | 3 | 0.5 | 3 | 0.5 |
| 43 | 21 | 1.75 | 1.75 | 1.75 | 1.75 | 1.75 |
| 15 | 22 | 0.5 | 3 | 3 | 3 | 0.5 |
| 24 | 23 | 3 | 3 | 3 | 0.5 | 3 |
| 39 | 24 | 1.75 | 1.75 | 1.75 | -1.22 | 1.75 |
| 5 | 25 | 0.5 | 0.5 | 3 | 0.5 | 0.5 |
| 34 | 26 | 4.72 | 1.75 | 1.75 | 1.75 | 1.75 |
| 36 | 27 | 1.75 | 4.72 | 1.75 | 1.75 | 1.75 |
| 37 | 28 | 1.75 | 1.75 | -1.22 | 1.75 | 1.75 |
| 9 | 29 | 0.5 | 0.5 | 0.5 | 3 | 0.5 |
| 6 | 30 | 3 | 0.5 | 3 | 0.5 | 0.5 |
| 18 | 31 | 3 | 0.5 | 0.5 | 0.5 | 3 |
| 13 | 32 | 0.5 | 0.5 | 3 | 3 | 0.5 |
| 30 | 33 | 3 | 0.5 | 3 | 3 | 3 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 31 | 34 | 0.5 | 3 | 3 | 3 | 3 |
| 44 | 35 | 1.75 | 1.75 | 1.75 | 1.75 | 1.75 |
| 27 | 36 | 0.5 | 3 | 0.5 | 3 | 3 |
| 32 | 37 | 3 | 3 | 3 | 3 | 3 |
| 45 | 38 | 1.75 | 1.75 | 1.75 | 1.75 | 1.75 |
| 14 | 39 | 3 | 0.5 | 3 | 3 | 0.5 |
| 2 | 40 | 3 | 0.5 | 0.5 | 0.5 | 0.5 |
| 46 | 41 | 1.75 | 1.75 | 1.75 | 1.75 | 1.75 |
| 26 | 42 | 3 | 0.5 | 0.5 | 3 | 3 |
| 21 | 43 | 0.5 | 0.5 | 3 | 0.5 | 3 |
| 10 | 44 | 3 | 0.5 | 0.5 | 3 | 0.5 |
| 8 | 45 | 3 | 3 | 3 | 0.5 | 0.5 |
| 16 | 46 | 3 | 3 | 3 | 3 | 0.5 |
| 50 | 47 | 1.75 | 1.75 | 1.75 | 1.75 | 1.75 |
| 38 | 48 | 1.75 | 1.75 | 4.72 | 1.75 | 1.75 |
| 49 | 49 | 1.75 | 1.75 | 1.75 | 1.75 | 1.75 |
| 42 | 50 | 1.75 | 1.75 | 1.75 | 1.75 | 4.72 |

### 5.2.7 Validation of RSM results

Flask level denitrification studies with *Thauera* sp.V14 was carried out as mentioned in chapter 5 section 5.2.4. Biofilm forming ability of *Thauera* sp. V14 was quantified using crystal violet staining. For biofilm quantification cover slips were immersed in the flasks for biofilm development. After 48 h of incubation the cover slips were rinsed five times with 3 ml of sterile PBS to remove any adhering planktonic cells. The biofilm formed was then stained with 3 ml of 1 % crystal violet for 45 min, rinsed five times with 3 ml of distilled water and destained with 70 % ethanol for 15 min. The absorbance of the decanted solution was measured in Tecan (InfiniteM200pro) microtiter plate reader at $OD_{595nm}$ (Srinandan et al., 2010).

### 5.2.8 Nitrate removal studies in continuous MBBR inoculated with *Thauera* sp.V14

For continuous MBBR studies *Thauera* sp.V14 was grown in PNB for 24 h. The absorbance was set at 0.5 $OD_{600nm}$. The cell pellet obtained after centrifugation at 8000 rpm for 7 min was washed twice with PBS and resuspended in the 90 ml PBS. This 90 ml of suspension of the *Thauera* sp. V14 was then added in the 810 ml of MM2 medium to make up the final volume of 900 ml and incubated at 37 °C under the static condition for 24 h and was used as an inoculum.

For continuous reactor studies, 10 L of synthetic effluent (MM2 medium) was continuously fed from the inlet tank to the reactor with the peristaltic pump (Masterflex®). 9 % of inoculum was added in the 10 L reactor containing carriers and biofilm was allowed to form on the carriers for 10 days. Studies were carried out with C/N ratio 0.3, HRT of 3 h and filling ratio 20 % of pall ring carrier (Optimized in chapter 3). pH-8, DO-0.1-0.8 mg $L^{-1}$ and 15-350 NTU turbidity were maintained in the dMBBR. Treated synthetic effluent was collected in the outlet tank and assayed for nitrate, nitrite, ammonia, pH, turbidity, biomass and DO at temperature 37°C. All the assays were performed according to section 2.2.13. Each reactor slot was repeated three times.

### 5.2.9 Quantification of abundance of *Thauera* sp.V14 on carriers

Abundance of *Thauera* in the biofilm developed inside dMBBR was quantified by using Real Time PCR (Applied Biosystems StepOne™ Real-Time PCR System). *Thauera* specific primers Forward: TGCATTGCTGCTCCGAAC and Reverse : CGCTCGTTG CGGGACTTAACC used to quantify abundance of *Thauera* in the MBBR were as per Loy et al., (2005).

### 5.2.10 SEM analysis

SEM of the biofilm developed on carriers of dMBBR was carried out to characterize biofilm morphology. Carriers before biofilm development and after biofilm development were taken from dMBBR. Then they were coated with gold and examined under SEM at accelerating voltage of 15kV (JEOL JSM-6380 LV at ERDA, Vadodara).

### 5.2.11 FTIR analysis

FTIR analysis was used to characterize the major components of the biofilm (Maruyama et al., 2001). Bound EPS extracted from biofilm biomass was dried at 70 °C for 48 h and the dry matter was analyzed in FTIR Spectrometer (FTIR Spectrometer: ALPHA at Department of chemistry, MSU Baroda).

### 5.2.12 Data Analysis

All the experiments were performed in triplicate. The statistical significance was determined using one way ANOVA analysis with Tukey's multiple comparison test. Error bars represent standard deviations in GraphPad Prism 6.0 (San Diegao, CA, USA).

## 5.3 Results and Discussion

The isolate *Thauera* sp.V14 was observed to be an efficient denitrifier and biofilm forming isolate. Bioaugmentation of denitrifying MBBR with consortium DC5 showed *Thauera* as a most persistent and dominant organism in developed denitrifying biofilm associated with carriers in continuous dMBBR. Therefore, in this chapter denitrification studies were carried out with *Thauera* sp.V14. Among two strains of consortium DC5, *Thauera* spp. V9 and V14, *Thauera* sp.V14 showed highest denitrification efficiency, biofilm forming ability and amyloid production ability compared to *Thauera* sp.V9. Moreover, the nitrate removal efficiency of *Thauera* sp. V14 was same as that of *Thauera* sp.V14 alone when both the strains were inoculated together and no enhancement in nitrate removal efficiency was observed. Therefore, further studies were focused on *Thauera* sp.V14.

### 5.3.1 Characterization of *Thauera* sp.V14

*Thauera* sp. V14 was characterized for various attributes needed for biofilm mode of growth. Auto-aggregation ability of *Thauera* increased gradually from 34 % at 1 h to 93 % at 8 h and hydrophobicity from 7.7 % at day 1 to 83.8 % at day 5 (Fig.5.1). *Thauera* sp.V14 showed 93 % auto-aggregation capacity and 83.8 % hydrophobicity, which is important aspect for the formation of biofilm in dMBBR (Fig. 5.1a, b). High auto-

aggregation index of *Thauera* sp.V14 indicates a strong tendency for cells to cluster and high hydrophobicity of isolate indicates better auto-aggregation ability. Auto-aggregation ability and hydrophobicity of bacteria are closely related to the aggregation and adhesion between the bacterial surface and between other bacteria (Hong et al., 2020). Bioaugmentation of bacteria with auto-aggregation ability and hydrophobicity adhere firmly and reduces the loss of bacteria from the wastewater treatment system (Wang et al., 2018). Auto-aggregation ability and hydrophobicity of *Thauera* sp.V14 was higher than *Enterobacter* sp. strain FL which showed 54.3 % at 48 h (Wang et al., 2018) and *Methylobacterium gregans* DC-1, showed 38.7 % at 72 h (Hong et al., 2019). These attributes suggested that *Thauera* could be appropriate organism for MBBR.

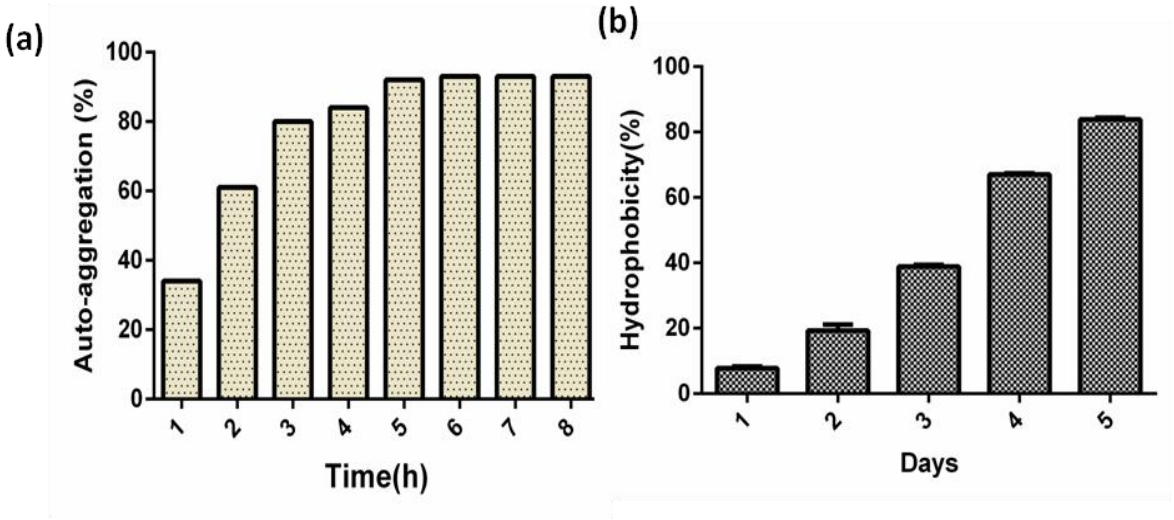



**Figure 5.1 (a) Auto aggregation ability (b) Hydrophobicity of *Thauera* sp.V14**

## 5.3.2 Effect of temperature on morphology and motility of *Thauera* sp.V14

Various environmental parameters such as temperature, pH and nutrients can influence formation and composition of the biofilm (Toyofuku et al., 2016). Biofilm developed in industrial cooling systems is influenced by temperature. It is one of the important parameter which can alter the metabolic rate (Gillooly et al., 2001), body size (Gardner et al., 2011; Sheridan and Bickford, 2011) and can exerts selective pressure on microbial communities (Lax et al., 2020) in wastewater treatment systems. Therefore, effect of temperature was also checked on *Thauera* sp.V14. *Thauera* colonies grown at different

temperatures showed red color due to binding of Congo red dye to the amyloid fibers present on the bacterial surface. The color of the colonies grown at 30 °C was light orange due to less amyloid production (Fig.5.2). Light red color was only localized in the raised wrinkles of the central core and boundary between the core and peripheral area. However, red color was observed in the peripheral area of colonies developed at 37–45 °C. Intense red colored colonies of *Thauera* indicate higher production of amyloid fibers at 40 °C (Fig.5.2). Swarming motility and amyloid both contribute to the biofilm formation process (Larsen et al., 2007; Larsen et al., 2008). Similar results were observed by Rajitha et al., (2021). They also reported that biofilm growth was increased at higher temperatures by increasing production of EPS and amyloid fibers in *Bacillus* sp.

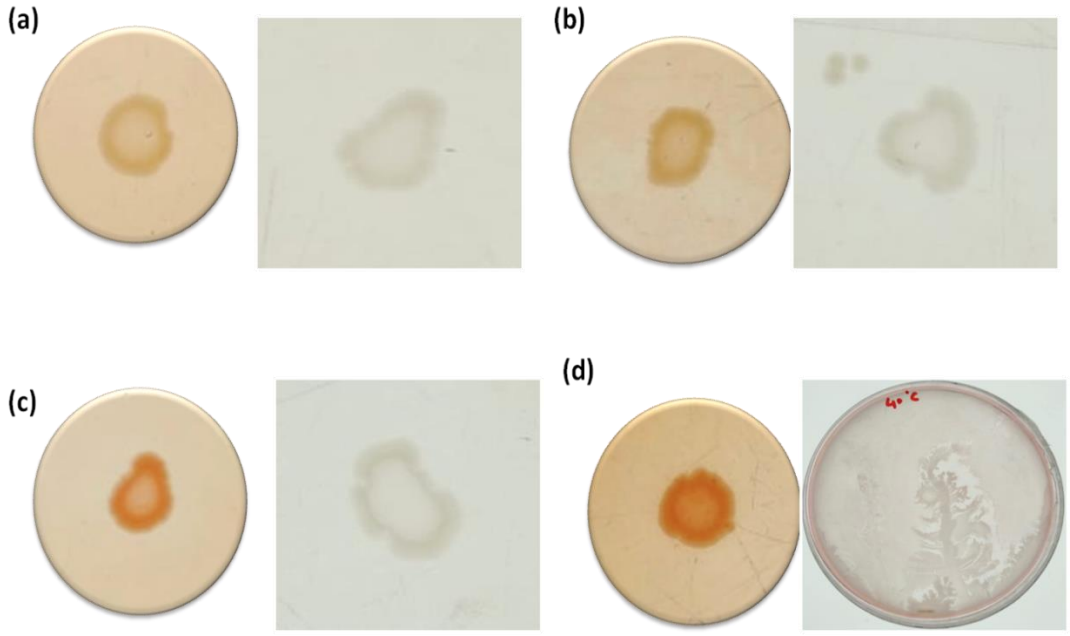



**Figure 5.2 Effect of temperature on morphology and swarming motility of *Thauera* sp.V14(a) 25 ºC, (b) 30 ºC ,(c) 37 ºC, (d) 40 ºC.**

## 5.3.3 Flask level denitrification studies with *Thauera* sp.V14

Flask level denitrification studies carried out with *Thauera* sp.V14 showed 100 % denitrification efficiency within 72 h with an initial nitrate concentration of 765.89 mg $L^{-1}$ while no nitrite and ammonia were accumulated in the medium (Fig.5.3). Denitrification efficiency of *Thauera* sp.V14 was higher compared to many organisms reported in the literature. Among them, *Acinetobacter haemolyticus* ZYL removed 100 %

nitrate at 36 h with initial concentration of 443 mg $L^{-1}$ (Wang et al., 2021), *Pseudomonas stutzeri* strain XL-2 removed 97.9 % of nitrate within 24 h with an initial concentration of 443 mg $L^{-1}$ (Zhao et al., 2018), *Pannonibacter phragmitetus* B1 reduced 98.77 % nitrate within 18 h with an initial nitrate concentration of 65.16 mg $L^{-1}$ (Bai et al., 2019), *Pseudomonas* sp. JQ-H3 was able to reduce 438.6 mg $L^{-1}$ of nitrate within 72 h (Wang et al., 2019c) and *Zoogloea* sp. N299 reduced 75.42 % nitrate within 72 h (Huang et al., 2015).

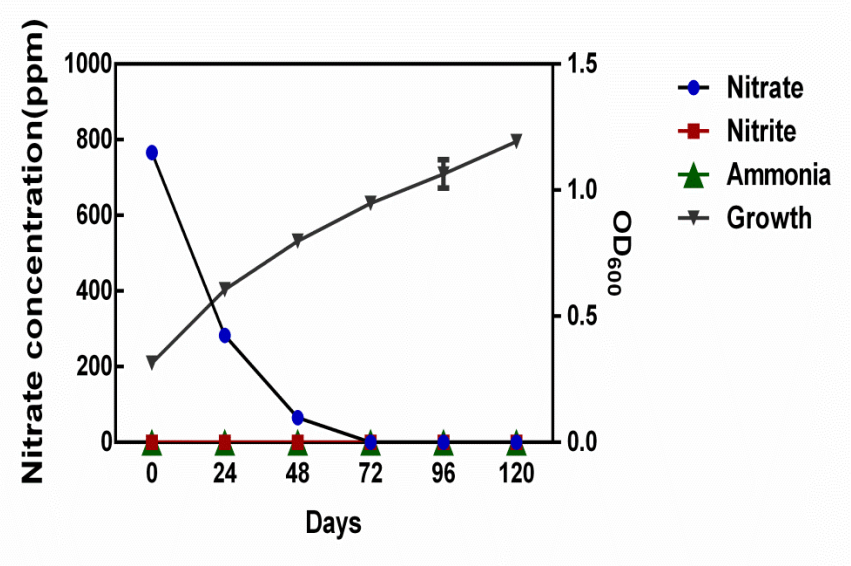



**Figure 5.3 Flask level denitrification studies with *Thauera* sp.V14**

## 5.3.4 Effect of different parameters affecting denitrification and biofilm formation of *Thauera* sp.V14 (One factor at a time (OFAT))

Effect of inoculum and metal ions on denitrification and biofilm of consortium DC5 was checked and has been discussed in detail in chapter 2. However, the parameters optimized for consortium DC5 were not optimum for *Thauera* sp. V14 as its performance was found to be low. Therefore, to start the studies the same parameters were again optimized for *Thauera* sp.V14.

### 5.3.4.1 Effect of Inoculum

As shown in Fig.5.4 as the inoculum size increased denitrification efficiency of *Thauera* sp.V14 was also increased. Inoculum size above 6 % increased denitrification efficiency whereas no significant difference was observed above 7 %. Inoculum size 9 % showed

high biofilm forming ability of *Thauera* sp.V14. This inoculum size is acceptable as industrial fermentations usually use inoculum volume less than 10 % (Zhou et al., 2018).

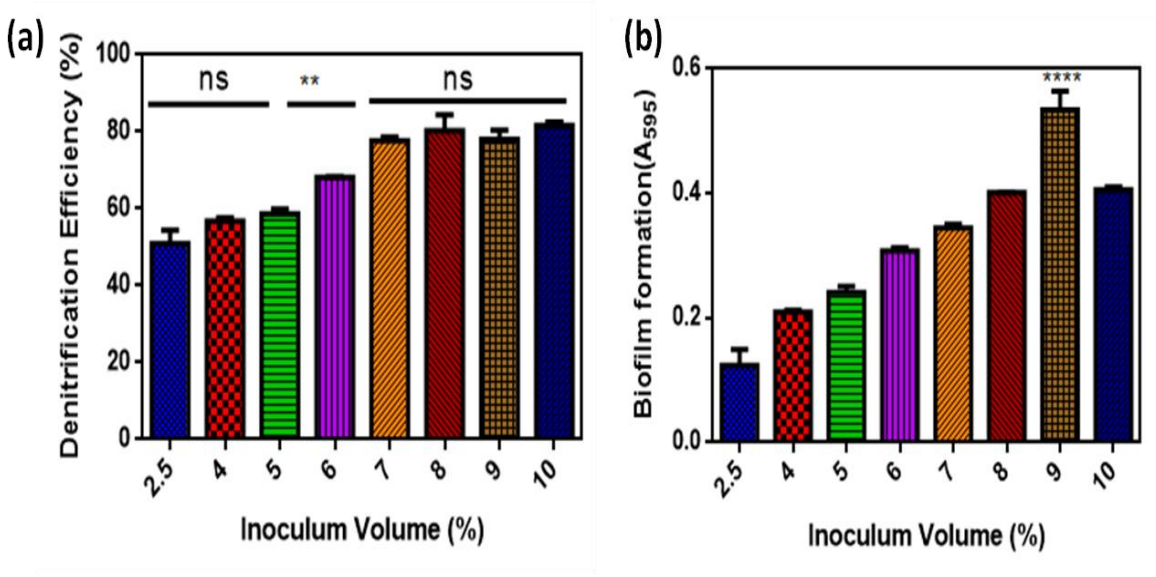



**Figure 5.4 Effect of inoculum on (a) Denitrification efficiency of *Thauera* sp.V14 (b) Biofilm of *Thauera* sp. V14.**
Statistical significance was analyzed using the one way ANOVA analysis. Error bars represent standard deviations, n=3. * $p<0.05$, ** $p<0.01$, ***$<0.001$, ****$<0.0001$.

### 5.3.4.2 Effect of metal ions

Divalent cations like $CaCl_2$ and $MgCl_2$ were reported to promote biofilm formation (Somerton et al., 2015; Haque et al., 2017). Fig. 5.5 showed that 10 mM and 9 mM concentration of calcium and magnesium showed highest biofilm formation. Whereas no significant effect was observed on denitrification efficiency of *Thauera* sp.V14. Metal ions contribute through bridging of cell surface and substratum and by decreasing charges on the surfaces. This enhances aggregation of cells and their ability to adhere to the substratum (Wang et al., 2018).

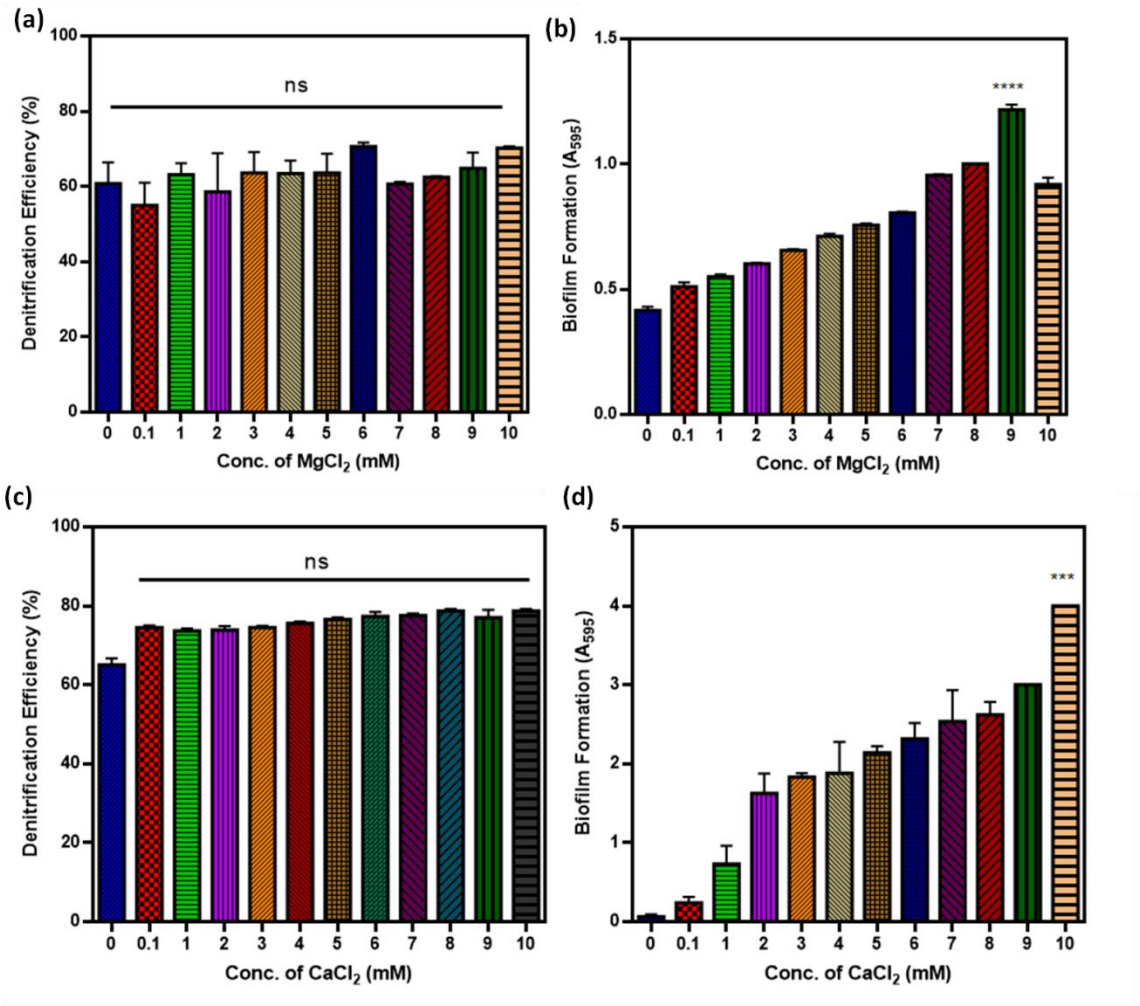



**Figure 5.5 Effect of metal ions on (a) Denitrification efficiency of *Thauera* sp.V14 in presence of $MgCl_2$ (b) Biofilm of *Thauera* sp. V14 in presence of $MgCl_2$ (c) Denitrification efficiency of *Thauera* sp.V14 in presence of $CaCl_2$ (d) Biofilm of *Thauera* sp. V14 in presence of $CaCl_2$.**

Error bars represent standard deviations, n=3. *p<0.05,**p<0.01,***<0.001, ****<0.0001.

### 5.3.4.3 Effect of purified amyloid concentration

Addition of purified amyloid protein increased the rate of biofilm formation and denitrification efficiency. Here, 10, 20, 30 mg/ml concentration of purified amyloid increased denitrification efficiency of *Thauera* sp.V14 but above 50 mg/ml denitrification efficiency was decreased. Biofilm forming ability of *Thauera* sp.V14 was decreased as the amyloid concentration increased (Fig.5.6).

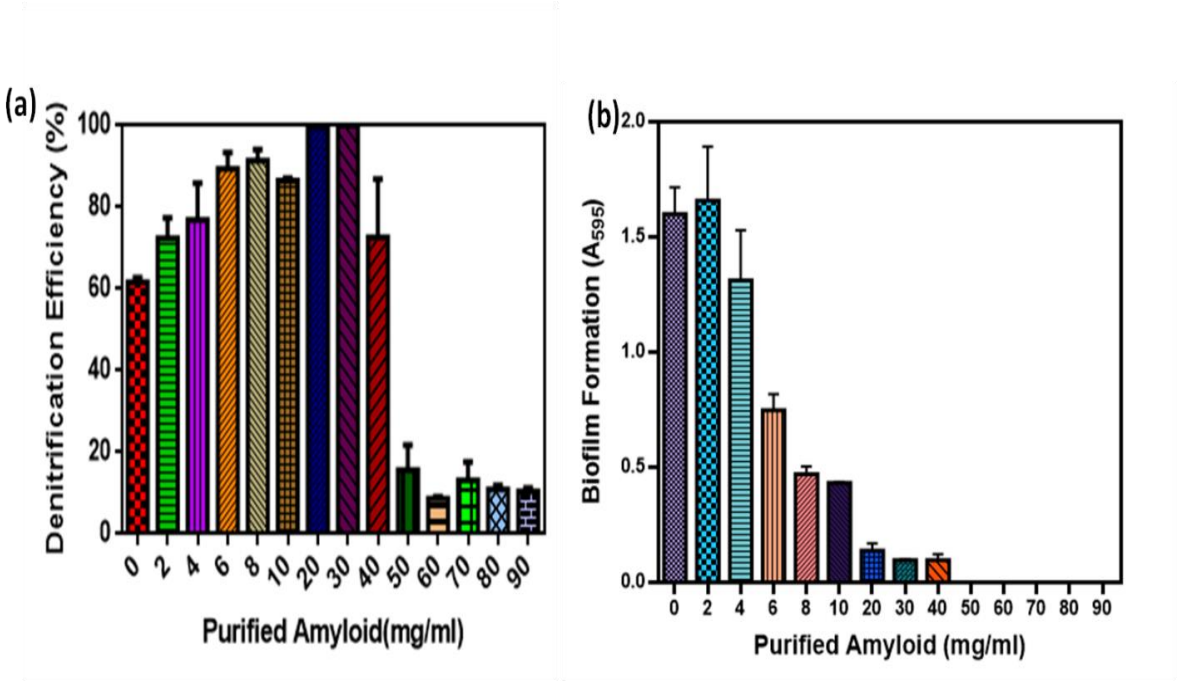



**Figure 5.6 Effect of Purified amyloid protein on (a) Denitrification efficiency of *Thauera* sp.V14 (b) Biofilm of *Thauera* sp. V14.**
Error bars represent standard deviations, n=3.

### 5.3.4.3 Effect of stress agents like Ethanol, NaCl and DMSO on biofilm and denitrification of *Thauera* sp.V14

Nutritional sources such as peptone, yeast extract and DMSO are known to increase the amyloid production (Lim et al., 2012; Markande and Nerurkar, 2021). Similarly ethanol and NaCl too has been found to have profound effect on amyloid production (Lim et al., 2012; Wang et al., 2020).

#### 5.3.4.3.1 Effect of NaCl

Sodium chloride has been demonstrated to increase biofilm consistency and wastewater treatment efficiency in biofilm based wastewater treatment systems (Wang et al., 2020). As the NaCl concentration increased denitrification efficiency and biofilm forming ability of *Thauera* sp. V14 were also increased. 0.5 % NaCl showed highest denitrification efficiency and biofilm forming ability.

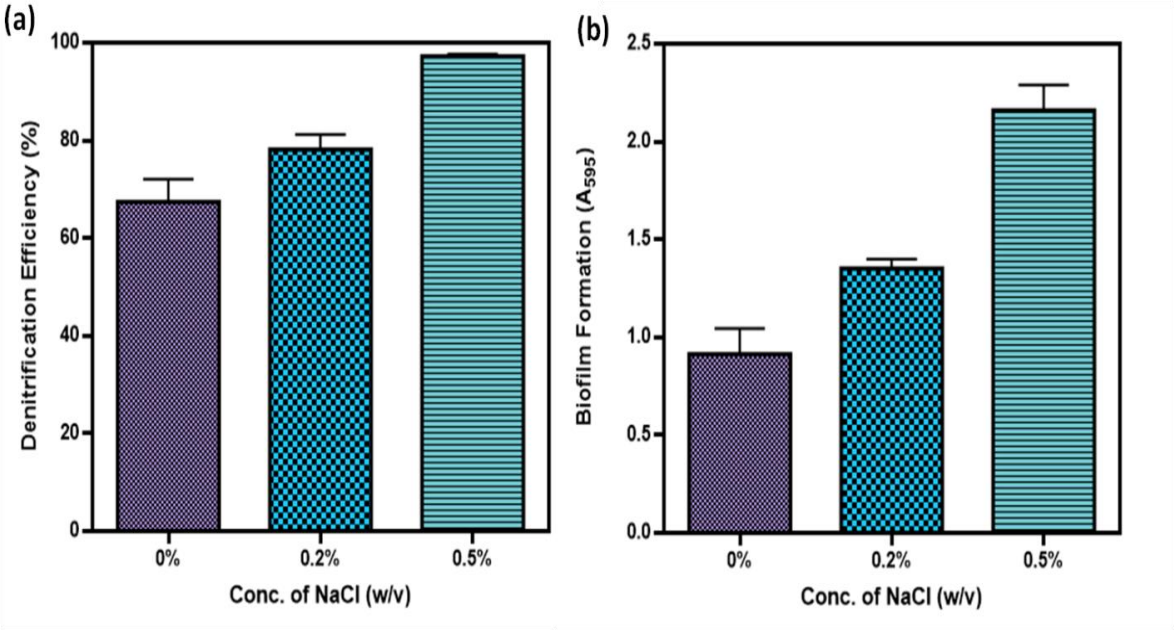



**Figure 5.7 Effect of NaCl on (a) Denitrification efficiency of *Thauera* sp.V14 (b) Biofilm of *Thauera* sp. V14.**
Error bars represent standard deviations, n=3.

### 5.3.4.3.2 Effect of DMSO

DMSO is known to upregulate amyloid formation in *E. coli*, however the mechanism remains to be elucidated (Lim et al., 2012). 0.05 % (v/v) concentration of DMSO showed highest denitrification efficiency (Fig.5.8). Rodriguez et al., (2021) reported that supplementation DMSO increased biofilm formation of *Shewanella algae* bacterial isolates. In *S. algae* DMSO acts as anaerobic electron acceptor for cellular respiration. Physiological link between biofilm formation and cellular respiration has been observed in *Pseudomonas aeruginosa*, *Burkholderia pseudomallei*.

DMSO can be lethal for the bacterial cells at higher concentration. However, at low concentration, it acts as stress agent.

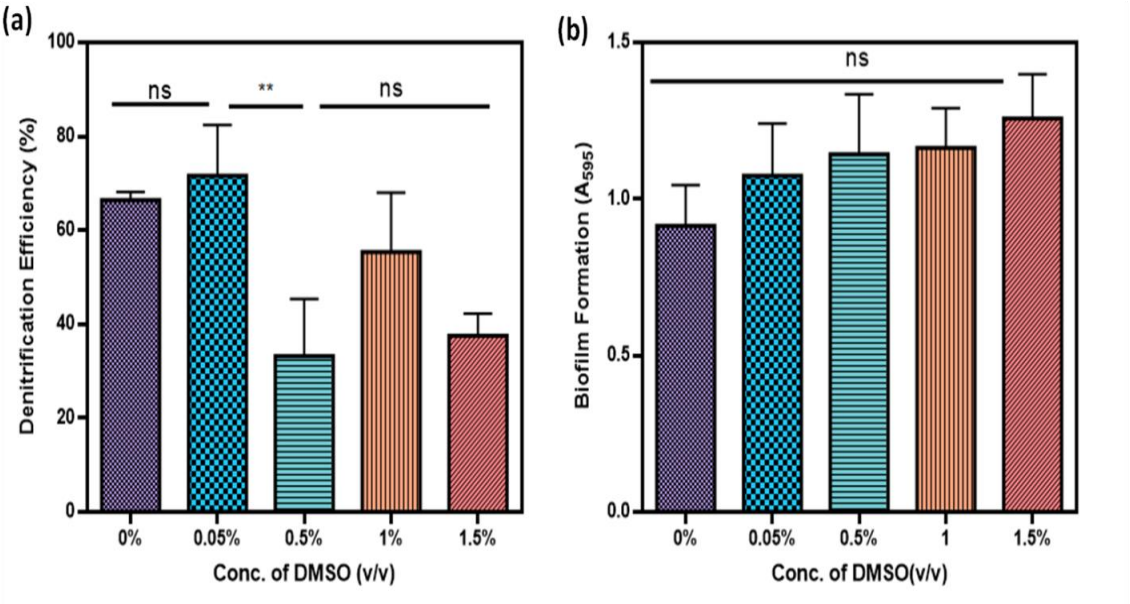



**Figure 5.8 Effect of DMSO on (a) Denitrification efficiency of *Thauera* sp.V14 (b) Biofilm of *Thauera* sp. V14.**
Error bars represent standard deviations, n=3.

### 5.3.4.3.3 Effect of Ethanol

Ethanol is also well known available carbon source for denitrification and at sub lethal concentrations it exerts stresses to initiate biofilm formation. It disrupts lipid membranes and can kill most bacteria. Sub-lethal concentrations exert stress, which initiate biofilm formation in *E. coli* which is due to the overexpression of curli amyloid genes (Lim et al., 2012). *Thauera* sp. V14 showed highest denitrification efficiency at 0.05 % whereas biofilm forming ability was highest at 0.5 % which was decreased at higher concentration (Fig. 5.9).

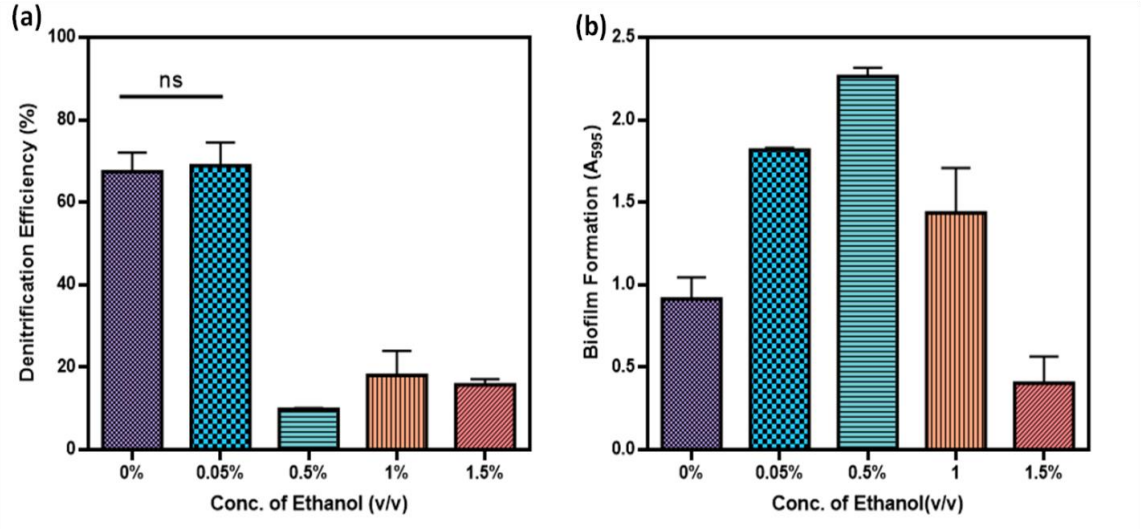



**Figure 5.9 Effect of Ethanol on (a) Denitrification efficiency of *Thauera* sp.V14 (b) Biofilm of *Thauera* sp. V14.**
Error bars represent standard deviations, n=3.

## 5.3.5 Medium optimization using Plackett-Burman Design

Plackett- Burman design was used to examine the roles of various factors that affect biofilm and denitrification efficiency of *Thauera* sp.V14. Plackett-Burman design is one of the widely used statistical method for understanding the significance of assortment of factors for e.g in present case significance of different factors on biofilm formation and denitrification efficiency of *Thauera* sp.V14. Experimental ranges of independent variables, namely, Peptone (A), Yeast extract (B), Purified amyloid (C), DMSO (D), Ethanol (E), NaCl (F), $MgCl_2$ (G) and $CaCl_2$ (H) that were used for the study were kept from 0.5 % (Low) to 3 % (High) concentration.The statistically significant variables were selected on the basis of F test. The critical value was determined using statistical F table. The critical value for 5 % significant level was 2.69, indicating that any ratio (of selected variable) above 2.69 will be statistically significant in its contribution for biofilm formation and denitrification efficiency.

The F value for each variable is given in the Table 5.3. Based on the F value Ethanol, $CaCl_2$ showed significant effect on biofilm formation whereas Peptone, DMSO, Yeast extract showed significant effect on the denitrification efficiency of *Thauera* sp.V14. Purified amyloid, NaCl and $MgCl_2$ showed no significant effect on biofilm formation and denitrification of *Thauera* sp.V14.

**Table 5.3 Statistical ANOVA analysis by using Plackett-Burman design**

| Response | Source | Sum of Squares | df | Mean Square | F-value | p-value |
|---|---|---|---|---|---|---|
| Denitrification | Peptone | 1493.43 | 1 | 1493.43 | 26.08 | 0.0145 |
| | Yeast extract | 529.21 | 1 | 529.21 | 9.24 | 0.0559 |
| | DMSO | 1806.88 | 1 | 1806.88 | 31.55 | 0.0112 |
| Biofilm formation | Ethanol | 10.25 | 1 | 10.25 | 9.50 | 0.0540 |
| | $CaCl_2$ | 3.63 | 1 | 3.63 | 3.36 | 0.16 |

### 5.3.6 Optimization of biofilm formation and denitrification efficiency of *Thauera* sp.V14 by Response surface methodology (RSM)

The response surface methodology uses experimental data to develop a mathematical equation that predicts the best response value. The Plackett-Burman analysis demonstrated the important nutrient factors that showed significant effect on biofilm formation and denitrification efficiency of *Thauera* sp.V14. RSM was used to find optimal concentrations of ethanol, $CaCl_2$, peptone, DMSO and yeast extract the factors that were indicated in Plackett-Burman design. The experimental runs for the central composite design were generated using Design expert software (Version 13). The accuracy of the equation was examined by ANOVA analysis of the quadratic model and lack of fit values (Table 5.4).

**Table 5.4 ANOVA analysis for media optimization for biofilm formation by *Thauera* sp.V14 using RSM**

| Source | Sum of Squares | df | Mean Square | F-value | p-value | |
|---|---|---|---|---|---|---|
| Model | 19.34 | 20 | 0.9669 | 2.93 | 0.0042 | significant |
| A-Peptone | 0.0292 | 1 | 0.0292 | 0.0883 | 0.7685 | |
| B-Yeast extract | 0.0008 | 1 | 0.0008 | 0.0024 | 0.9612 | |
| C- | 4.02 | 1 | 4.02 | 12.17 | 0.0016 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| DMSO | | | | | | |
| D-Ethanol | 0.9354 | 1 | 0.9354 | 2.83 | 0.1031 | |
| E-$CaCl_2$ | 5.43 | 1 | 5.43 | 16.43 | 0.0003 | |
| AB | 0.3924 | 1 | 0.3924 | 1.19 | 0.2846 | |
| AC | 0.0022 | 1 | 0.0022 | 0.0066 | 0.9359 | |
| AD | 0.3633 | 1 | 0.3633 | 1.10 | 0.3029 | |
| AE | 0.6350 | 1 | 0.6350 | 1.92 | 0.1761 | |
| BC | 0.1562 | 1 | 0.1562 | 0.4730 | 0.4971 | |
| BD | 0.1478 | 1 | 0.1478 | 0.4474 | 0.5089 | |
| BE | 0.0266 | 1 | 0.0266 | 0.0806 | 0.7785 | |
| CD | 0.2147 | 1 | 0.2147 | 0.6501 | 0.4266 | |
| CE | 1.71 | 1 | 1.71 | 5.19 | 0.0303 | |
| DE | 2.98 | 1 | 2.98 | 9.02 | 0.0055 | |
| $A^2$ | 0.5094 | 1 | 0.5094 | 1.54 | 0.2242 | |
| $B^2$ | 0.1064 | 1 | 0.1064 | 0.3221 | 0.5747 | |
| $C^2$ | 1.11 | 1 | 1.11 | 3.37 | 0.0765 | |
| $D^2$ | 0.2109 | 1 | 0.2109 | 0.6388 | 0.4307 | |
| $E^2$ | 1.21 | 1 | 1.21 | 3.65 | 0.0660 | |
| Residual | 9.58 | 29 | 0.3302 | | | |
| Lack of Fit | 9.53 | 22 | 0.4333 | 69.05 | < 0.0001 | significant |

P value < 0.05 suggested that DMSO (C), $CaCl_2$ (E) showed significant effect on biofilm formation whereas Peptone (A), Yeast extract (B) and Ethanol (D) showed P value > 0.05 suggesting its non- significant effect on biofilm formation of *Thauera* sp.V14. $CaCl_2$ showed significant effect on denitrification efficiency of *Thauera* sp.V14 whereas DMSO, Peptone, Yeast extract, Ethanol did not show any effect.

**Table 5.5 ANOVA analysis for media optimization for denitrification by *Thauera* sp.V14 using RSM**

| Source | Sum of Squares | df | Mean Square | F-value | p-value | |
|---|---|---|---|---|---|---|
| Model | 3806.49 | 5 | 761.30 | 1.08 | 0.3851 | not significant |
| A-Peptone | 128.37 | 1 | 128.37 | 0.1819 | 0.6718 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| B-Yeast extract | 467.26 | 1 | 467.26 | 0.6622 | 0.4202 | |
| C-DMSO | 54.16 | 1 | 54.16 | 0.0768 | 0.7830 | |
| D-Ethanol | 44.09 | 1 | 44.09 | 0.0625 | 0.8038 | |
| E-$CaCl_2$ | 3112.60 | 1 | 3112.60 | 4.41 | 0.0415 | |
| Residual | 31044.87 | 44 | 705.57 | | | |
| Lack of Fit | 27679.86 | 37 | 748.10 | 1.56 | 0.2821 | not significant |

**5.3.6.1 Optimization of process variables**

Three dimensional response surface plots were used to study the interaction effect of different process variables on biofilm formation and denitrification of *Thauera* sp.V14. Results of RSM suggested that the response yielded a linear model as there was no interaction seen among the components for biofilm formation and denitrification efficiency of *Thauera* sp.V14. DMSO as well as $CaCl_2$ were found to be most significant components influencing biofilm and denitrification respectively of *Thauera* sp.V14 with p-values of $<0.05$. Fig. 5.10 b, c shows that in presence of DMSO and $CaCl_2$ biofilm forming ability of *Thauera* sp.V14 increased. As the DMSO concentration increased from 0.5 % to 3 % biofilm forming ability of *Thauera* sp.V14 was also increased (Fig.5.10b). In case of $CaCl_2$ as the concentration increased biofilm forming ability of *Thauera* sp.V14 decreased (Fig.5.10c). Yeast extract, Peptone, Ethanol did not show any effect on biofilm forming ability of *Thauera* sp.V14 (Fig.5.10a, b, c).

**(a)**

Factor Coding: Actual

**Biofilm (OD 595nm)**

0 3.5662

X1 = A

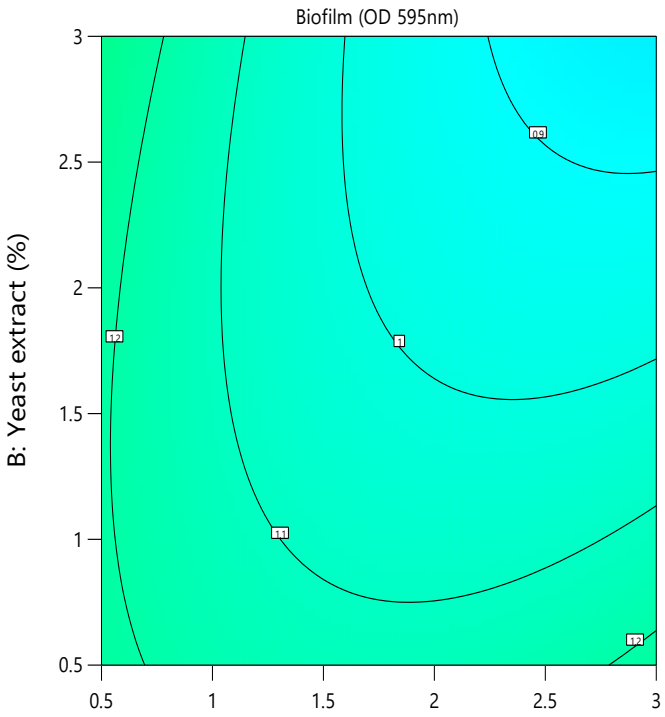
X2 = B

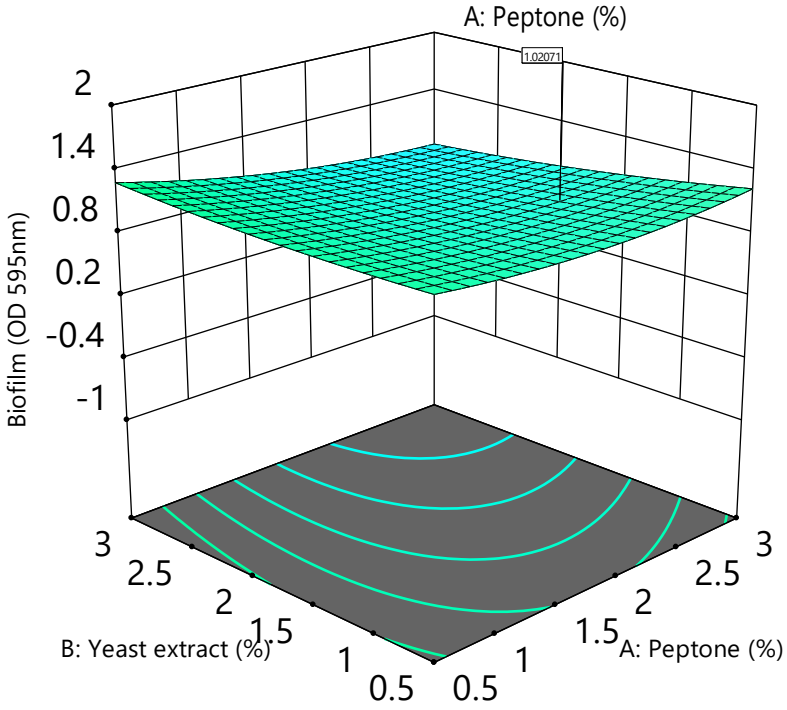
**Actual Factors**

C = 2.6

D = 1.75

E = 0.5



**(b)**

Factor Coding: Actual

**Biofilm (OD 595nm)**

0 3.5662

X1 = C

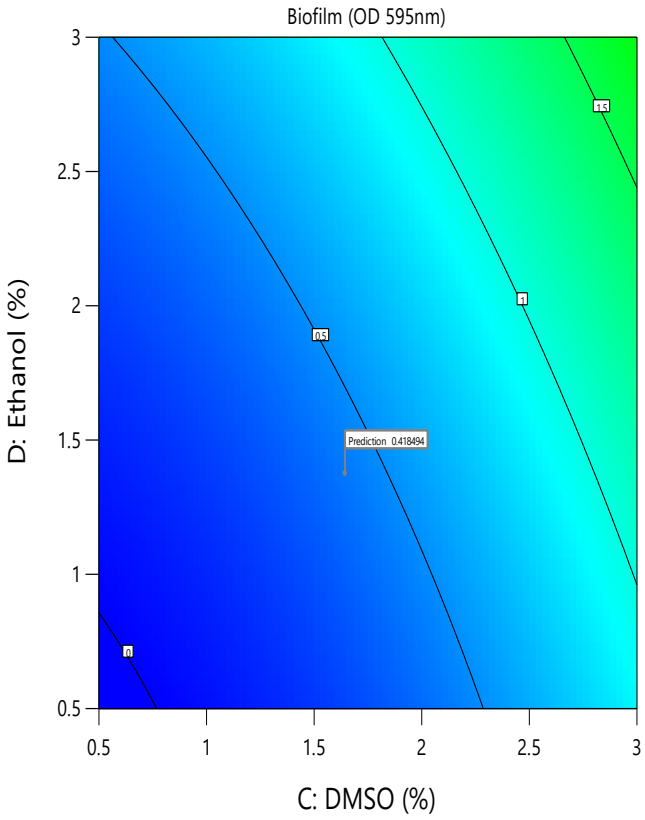
X2 = D

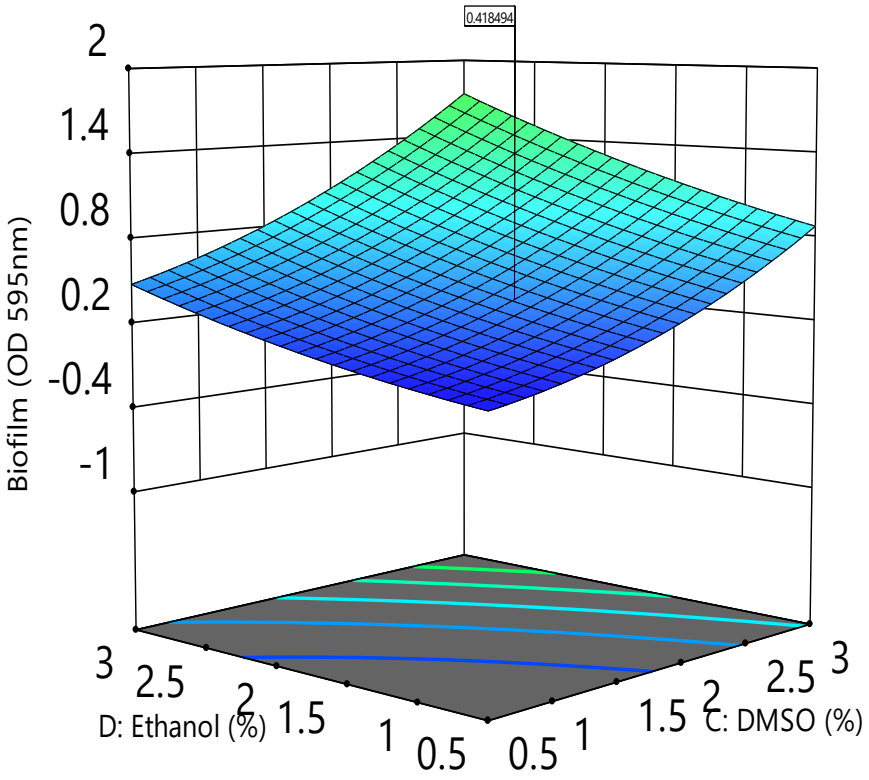
**Actual Factors**

A = 3

B = 1.75

E = 0.5



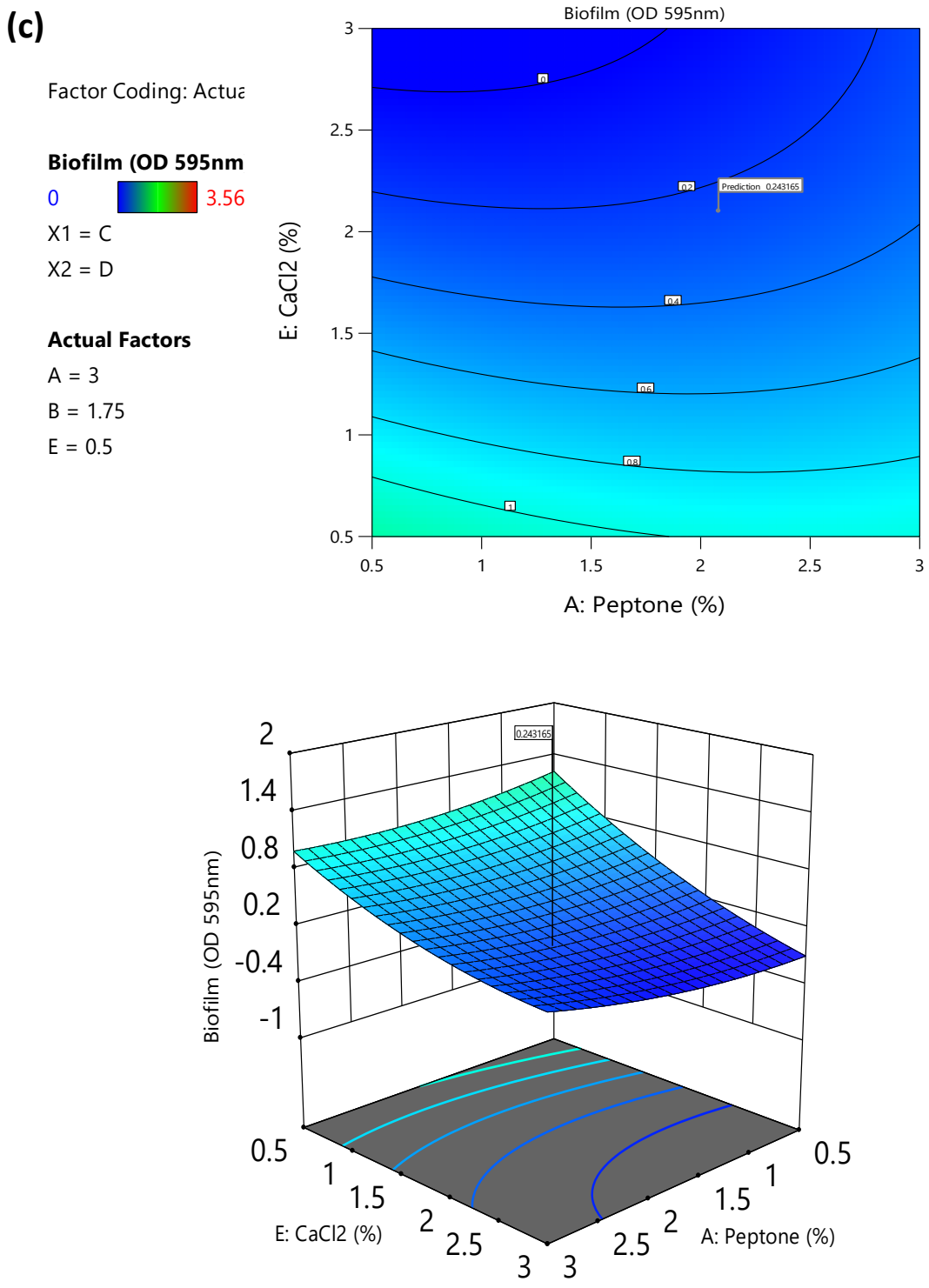



**Figure 5.10 Contour and 3-D graphs of (a) Effect of Yeast extract and Peptone (b) Effect of Ethanol and DMSO (c) Effect of $CaCl_2$ and Peptone on biofilm forming ability of *Thauera* sp.V14.**

**(a)**

Factor Coding: Actual

**Denitrification (%)**

Design Points

0 81.5176

X1 = E

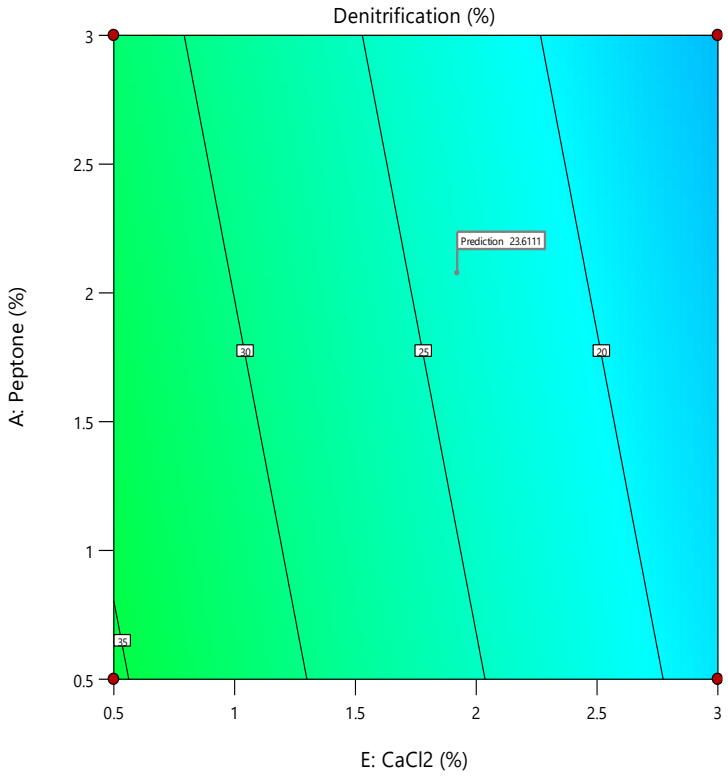
X2 = A

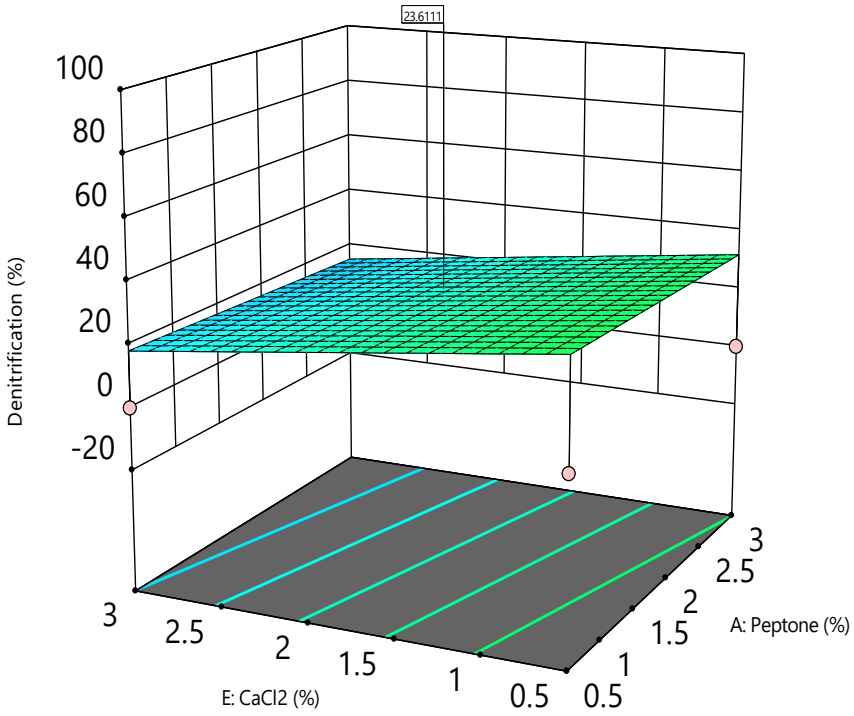
**Actual Factors**

B = 3

C = 3

D = 3



**(b)** Factor Coding: Actual

**Denitrification (%)**

0 81.5176

X1 = C

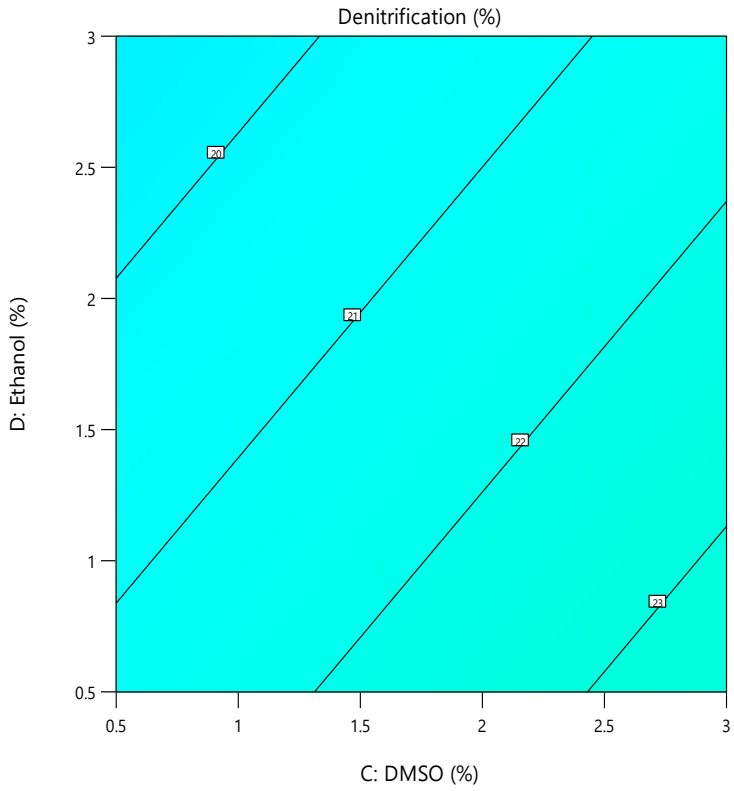
X2 = D

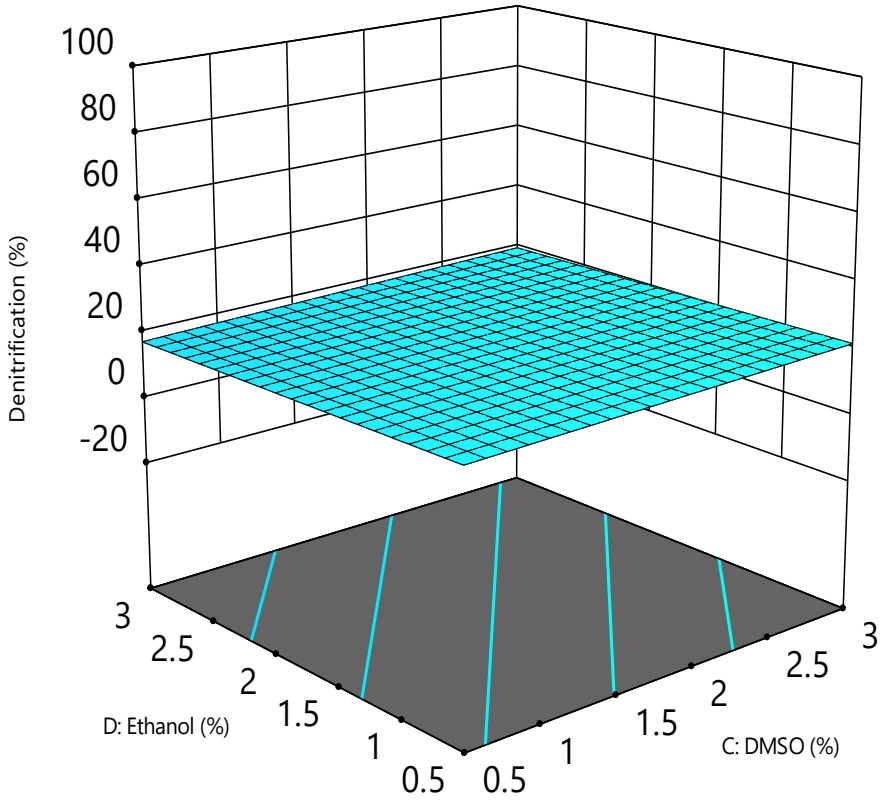
**Actual Factors**

A = 1.75

B = 3

E = 2.3



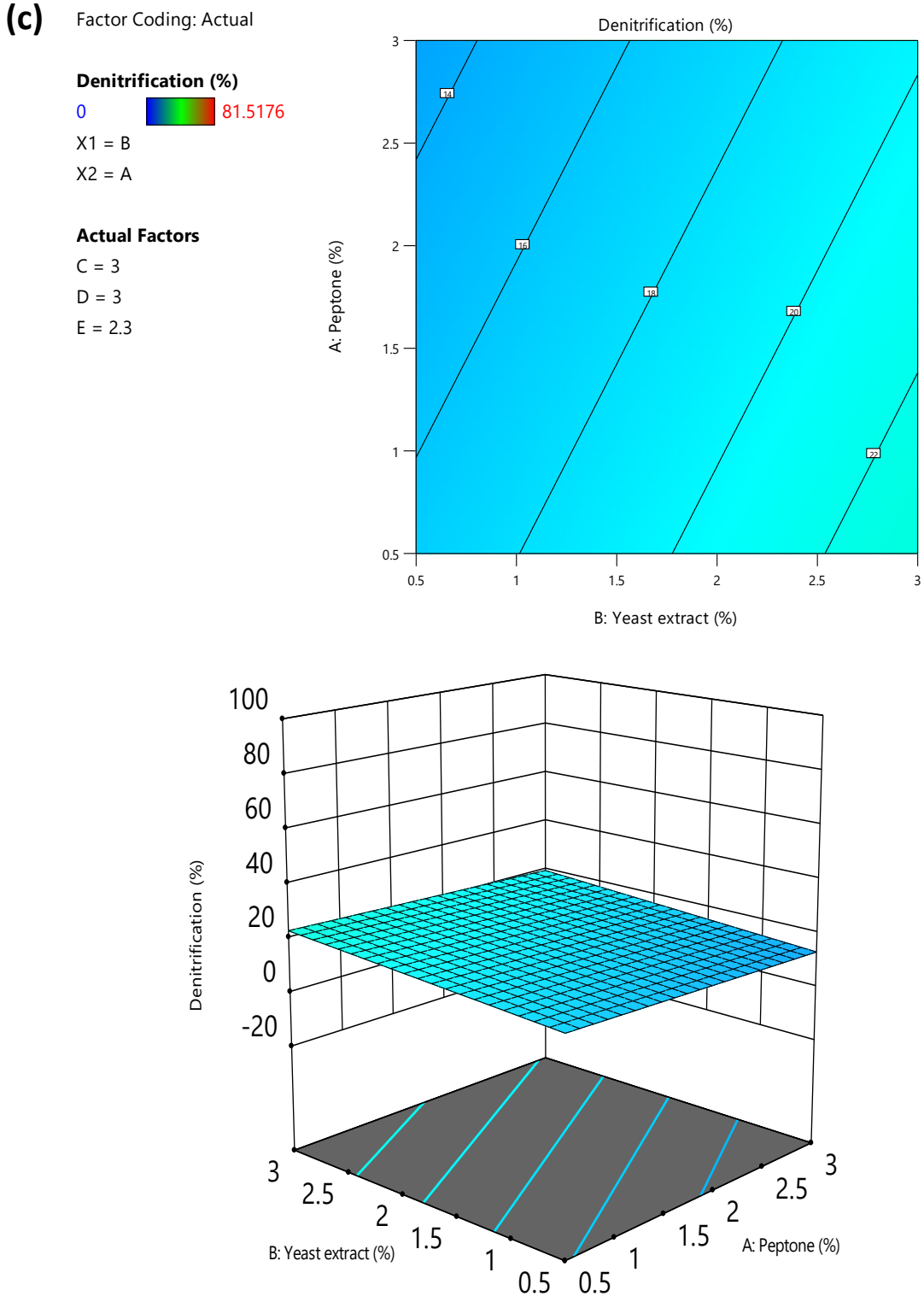



**Figure 5.11 Contour and 3-D graphs of (a) Effect of Peptone and $CaCl_2$ (b) Effect of Ethanol and DMSO (c) Effect of Peptone and Yeast extract on denitrification ability of *Thauera* sp.V14.**

Fig.5.11a shows that in presence of $CaCl_2$ denitrification ability of *Thauera* sp.V14 increased. As the $CaCl_2$ concentration increased from 0.5 % to 3 % denitrification efficiency of *Thauera* sp.V14 was decreased (Fig.5.11a). Yeast extract, Peptone, Ethanol and DMSO did not show any effect on biofilm forming ability of *Thauera* sp.V14 (Fig.5.11a, b, c). Significant parameters affecting biofilm formation and denitrification were found to be DMSO (3 %) and $CaCl_2$ (0.5 %) using RSM.

### 5.3.7 Validation of optimized results of RSM

To validate results of RSM flask level denitrification studies were carried out with *Thauera* sp.V14 with optimized parameters (DMSO (3 %) and $CaCl_2$ (0.5 %)). Biofilm was also quantified from the same flasks. Optimized media showed 100 % denitrification efficiency with *Thauera* sp.V14 and increased biofilm forming ability up to 56 % as compared to biofilm formed in MM2 media (Fig.5.12).

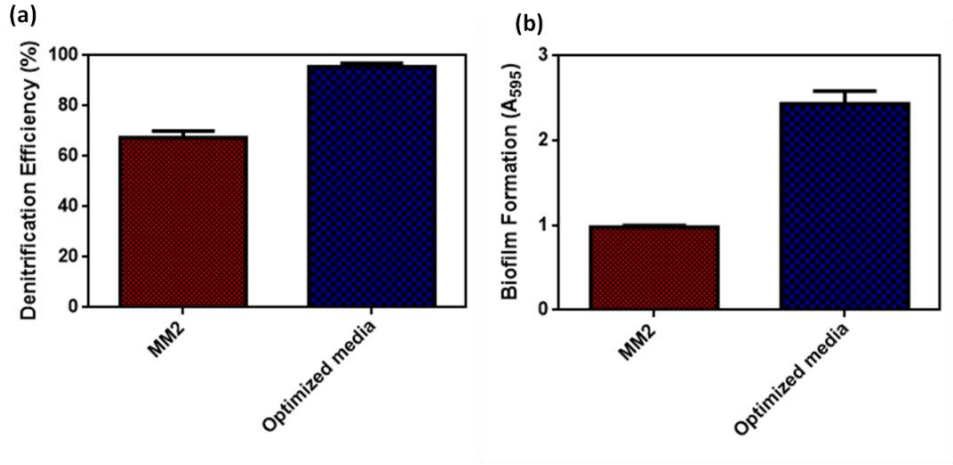



**Figure 5.12 Validation of RSM results at flask level (a) Denitrification efficiency of *Thauera* V.14, (b) Biofilm forming ability of *Thauera* sp.V14**

### 5.3.8 Nitrate removal studies in continuous dMBBR with *Thauera* sp .V14

To check the denitrification ability of the *Thauera* sp.V14 in the dMBBR different concentration of nitrate 620, 744, 930, 1500 and 2400 mg $L^{-1}$ was added in the influent of synthetic wastewater. As shown in Fig.5.13 denitrification efficiency in the dMBBR was 91 %, 90 %, 76 %, 66 % and 60 % amounting to 620, 744, 930, 1500 and 2400 mg $L^{-1}$ of nitrate concentration, while every time COD reduction was below stipulated

permissible range i.e. 250 mg $L^{-1}$. No nitrite and ammonia were detected in the dMBBR. Abundance of *Thauera* sp.V14 in the biofilm developed in MBBR as detected by RTPCR was $2\times10^8$ copy number /µl of the sample suggesting that *Thauera* was predominantly present in dMBBR and it can be concluded that probably it was contributing majorly to denitrification.

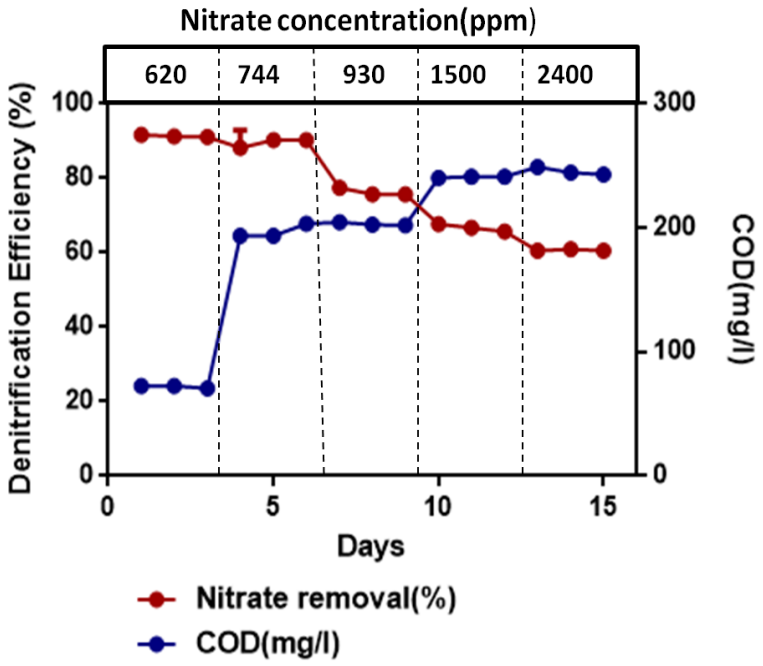



**Figure 5.13 Nitrate removal studies in continuous dMBBR inoculated with *Thauera* sp.V14**

## 5.3.9 Characterization of carrier associated *Thauera* biofilm developed in dMBBR

EPS components of the biofilm developed in dMBBR showed a broad absorption region between 3418 $cm^{-1}$ assigned to the O–H bond in hydroxyl functional groups. It confirmed the presence of the hydrogen bond of amines and alcohols (or phenols) in the biofilm. Small peaks of 2851, 2921$cm^{-1}$ were due to fatty acids and esters. Broad peak at 1426 $cm^{-1}$ was due to the presence of amides I and II. Peak at 1082 $cm^{-1}$ showed the presence of carbohydrates. Results of FTIR analysis indicate that EPS of *Thauera* showed the presence of Carbohydrates, Proteins and Lipid components in the carrier-associated biofilm developed in the dMBBR (Fig.5.14a). The biofilm morphology developed on the Pall ring carrier was examined using the SEM images. Fig.5.14b

represents the SEM images of the carriers before biofilm formation and after biofilm formation on Pall ring carriers. Carriers after biofilm formation showed high bacterial density and biofilm was dominated by rod shaped bacteria. Shen et al., (2013) also showed rod-shaped bacteria as the most predominant species in the denitrifying bioreactors.

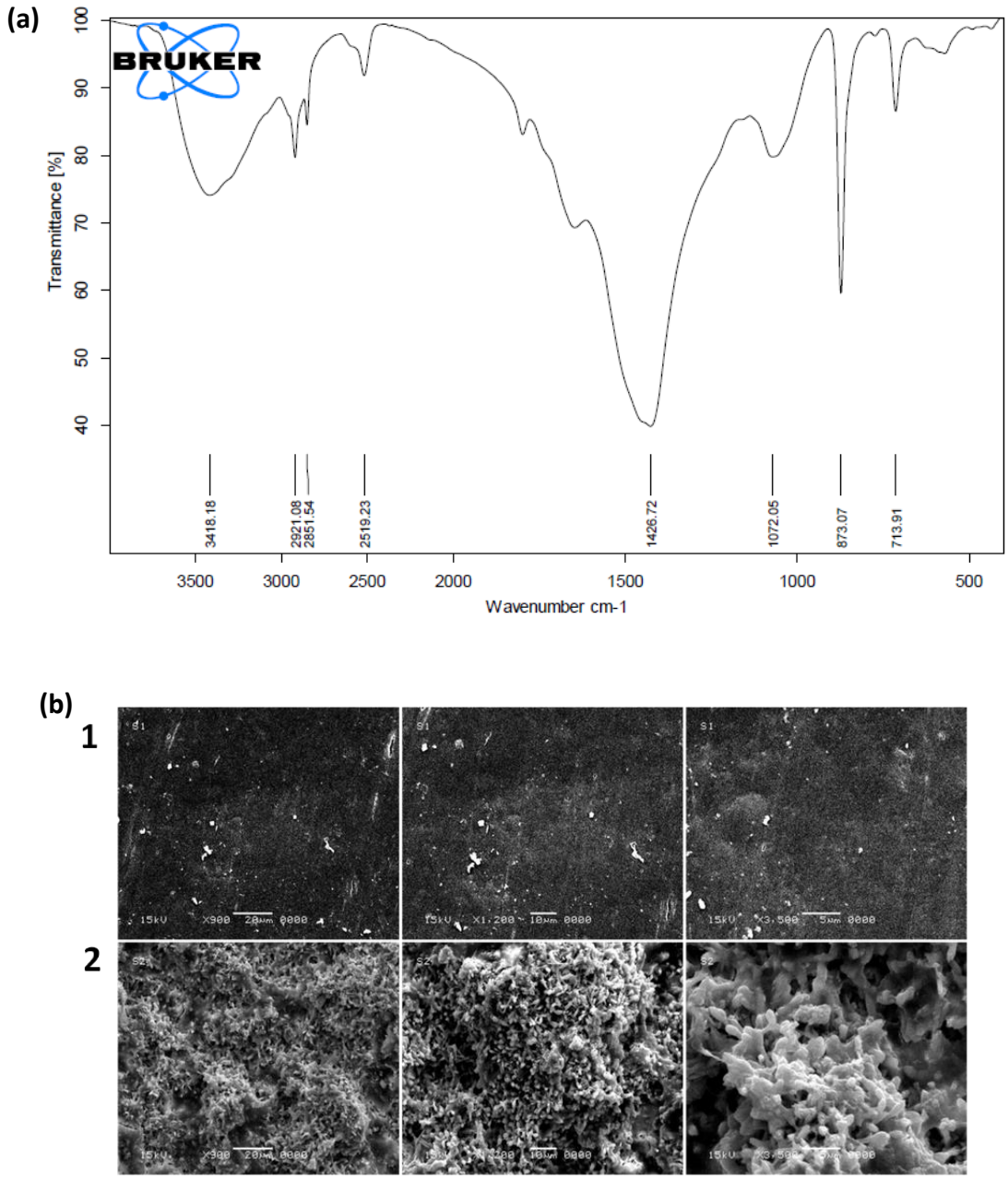



**Figure 5.14 (a) FTIR of the biofilm developed on the carrier, (b) SEM image of the surface of the carrier before and after biofilm development in dMBBR developed with *Thauera* sp.V14.**

Studies in this chapter revealed that *Thauera* sp.V14 with strong auto-aggregation ability, hydrophobicity, high denitrification efficiency and good biofilm forming traits can be used for the denitrification of nitrate containing wastewater. Plackett-Burman analysis and RSM results showed DMSO and $CaCl_2$ as major factors affecting denitrification efficiency and biofilm forming ability of *Thauera* sp.V14. Nitrate removal studies in dMBBR with *Thauera* suggested that it could efficiently remove nitrate and COD from wastewater without accumulation of $NO_2$ and $NH_4$. In a broader perspective, denitrifiers belonging to genus *Thauera* were found not only persistent and dominant in dMBBR but possibly could be majorly responsible for nitrate removal.