The Effect of Environmental parameters on Vitamin B_2 Production from Hydrocarbons by *Aspergillus awamori* NRRL 3112

Vaishnavi M. and David K. Daniel[1]

Bioprocess Engineering Laboratory, Chemical Engineering Division, SMBS, VIT University, Vellore-632014, Tamil Nadu, India.

ABSTRACT

The fungus *Aspergillus awamori* NRRL 3112 was tested for its ability to grow on crude oil from the petroleum industry. The potential of *Aspergillus awamori* NRRL 3112 to utilize the hydrocarbons in the crude oil and produce Vitamin B_2 was measured. The fungus exhibited a growth rate of 0.251 per day in the media containing the crude oil. A maximum Vitamin B_2 concentration of 143.75 μg/ml was obtained when *Aspergillus awamori* NRRL 3112 was incubated in medium containing the crude oil for 20 days. An initial pH value of 5.0 was found to be the optimum for the growth of *Aspergillus awamori* NRRL 3112 and for Vitamin B_2 production. Increased temperatures were found to decrease the Vitamin B_2 yields.

Introduction

Riboflavin (Vitamin B_2) is an easily absorbed micronutrient found in a variety of food sources like milk, cheese, green leafy vegetables, tomatoes, mushrooms and almonds. It is useful in human nutrition and therapy. The chemical synthesis of riboflavin involves four steps, starting from conversion of glucose to ribose, ribose to riboside by xylidine, which is then hydrogenated to ribammine. This reacts with phenyl diazonium salt to produce phenyl azo ribtiyl amine, which is crystallised, dried and condensed to give riboflavin (Stahmann et. al, 2000). On the contrary, microbial synthesis involving a single stage fermentative route is considered simple and cost effective. It comprises of use of simple stirred tank reactors with fixed agitating mechanisms, unlike sophisticated equipments that are used in chemical synthesis. The yield of the chemical process is around 60% and the purity of the product is 96%. On the other hand, the yield from the microbial synthesis is 80% and the purity is 98%, depending on the choice of the down streaming adopted. Further the chemical process requires 25% more energy than the microbial process (Van loon et. al, 1996; Vandamme, 1992). One of the major concerns in the microbial synthesis of riboflavin is the cost of the nutrients that are employed for the growth of the microorganisms. Hence efforts are now on for identifying cheaper substrates and the potentiality of the microorganisms to act on these kinds of substrates. In this context crude oil a mixture of various hydrocarbons is regarded as a potential substrate. Therefore in the present work, the fungus *Aspergillus awamori* NRRL 3112 was tested for its ability to grow on crude oil. This work aims to generate a bioremediation process for handling crude oil spills during its transport with simultaneous production of riboflavin.

[1] Corresponding Author:
Professor
Chemical Engineering Division
SMBS, VIT University
Vellore-632014 Tamil Nadu
India
Phone: 91 416 2202515/2202538
Fax: 91 416 2243092
E-mail: davidkdaniel@vit.ac.in

Materials and Methods

Microorganism and inoculum development

Aspergillus awamori NRRL 3112 donated by Northern Regional Research Laboratory, Illinois, USA was used throughout this work. It was grown on potato dextrose agar medium and sub cultured fortnightly. The fungus was then acclimatized for growth in the crude oil in mineral medium with the following composition. $(NH_4)_2SO_4$ – 3.75 g/l, $NH_4H_2PO_4$ – 3.75 g/l, KH_2PO_4 – 2.5 g/l, K_2HPO_4 – 2.5g/l, $MgSO_4.7H_2O$ – 0.5 g/l. The pH of the medium was maintained at 6.4 prior autoclaving (120^{o}C for 20 minutes) unless otherwise mentioned. Crude oil was supplied by Deva Drill Tech Ltd., Raigad, Maharashtra.

Growth studies

The mineral medium with the crude oil was inoculated with acclimatized inoculum (10 % v/v) in erlenmeyer flasks and incubated at 25^{o}C and 120 rpm in an incubator shaker. Samples were withdrawn twice everyday for 20 days. 5 ml of sample was centrifuged at 800 rpm for 10 minutes. The supernatant was separated and retained for further testing and the cells were scraped off into a filter paper and dried in a hot air oven overnight maintained at 60^{o}C. The cell mass was estimated from the difference in weights after the drying period.

Estimation of Riboflavin

In order to quantify the amount of riboflavin produced, a standard solution containing 20 µg/ml of riboflavin with 200 ml of 5N acetic acid and 2 ml of 1N NaOH was prepared in a1000 ml standard flask. Varying concentrations of this standard solution were prepared and the absorbance at 267 nm was determined using a UV-visible spectrophotometer. A standard plot of absorbance versus concentration for riboflavin was constructed and used for estimating the riboflavin content of the samples drawn at regular intervals. The percentage of riboflavin present in the sample was estimated using the relation

$$\%Riboflavin = 100 \times \frac{A_S}{A_R} \times \frac{W_R}{W_S}$$

where

$A_S = Absorbance\,of\ the\,sample$

$W_S = Weight\,of\ riboflavin, mg$

$A_R = Absorbance\,of\ the\,reference$

$W_R = Weight\,of\ riboflavin\,in\,reference, mg$

Results and Discussion

Growth Studies

The fungus was grown in the mineral medium containing the crude oil as the carbon source and the growth in this medium was compared with that in the potato dextrose medium. Fig 1 depicts the growth curve of *Aspergillus awamori* NRRL 3112 in both media. The maximum cell mass was 16.6 g/l on the seventh day of cultivation with a specific growth rate of 0.312 per day in the potato dextrose medium. In the crude oil medium, the maximum cell mass was only 8.6 g/l on the sixth day. The growth was examined for a time period of 20 days during which the stationary phase continued until the fifteenth day beyond which death phase occurred. The microscopic view of the mycelial mass in potato dextrose medium is shown in Fig 2. Considerable growth was observed in the medium with the crude oil, however the lag phase was found to be increased with an initiation of the stationary phase on the eighth day of growth. This observation of increase in the lag phase was probably due to the presence of the crude in the medium which ultimately favoured the secondary metabolic phase. Brown et al., (1957) have demonstrated that the presence of hydrocarbons in such medium led to an early secondary metabolic stage.

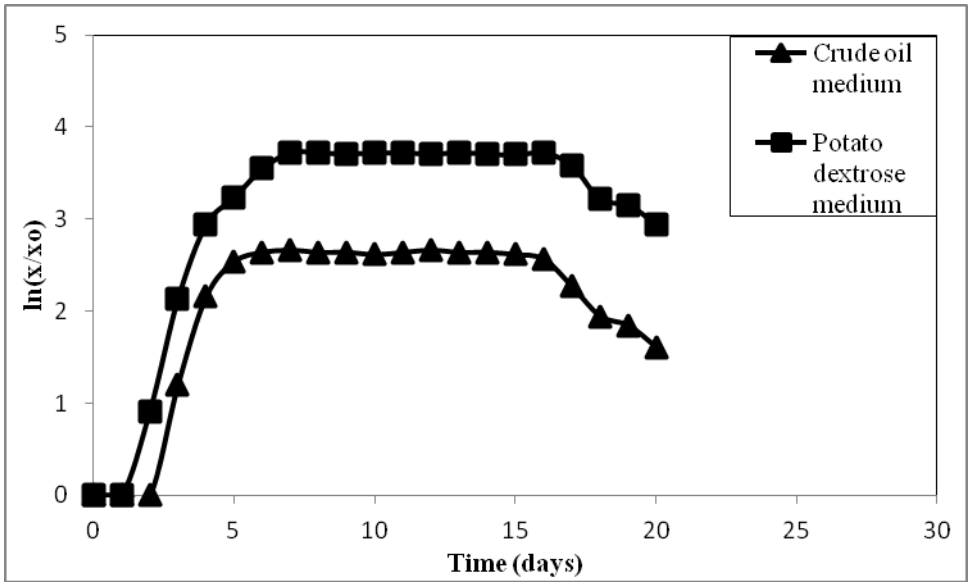

Fig 1: Growth of *Aspergillus awamori* NRRL 3112 in potato dextrose and crude oil medium

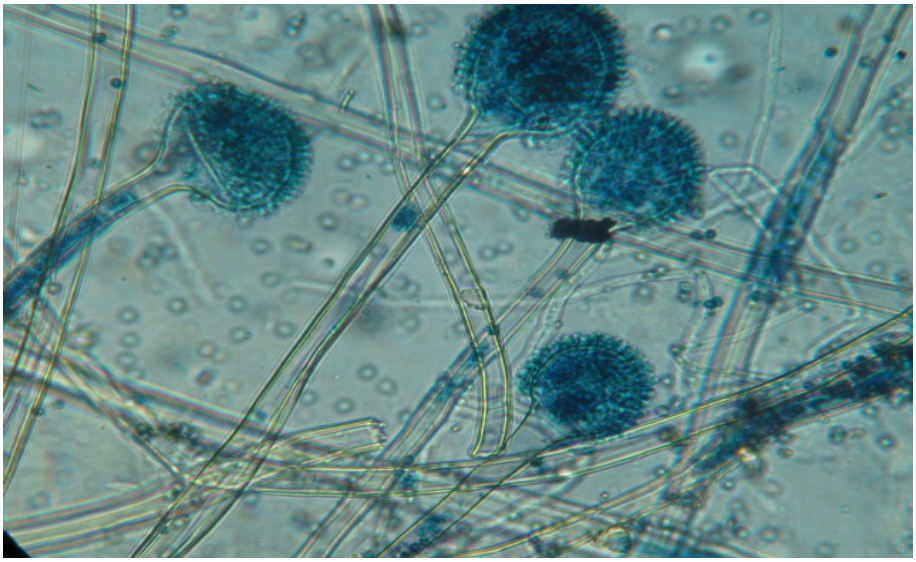

Fig.2. *A.. awamori* NRRL 3112 grown in potato dextrose medium (40X magnification)

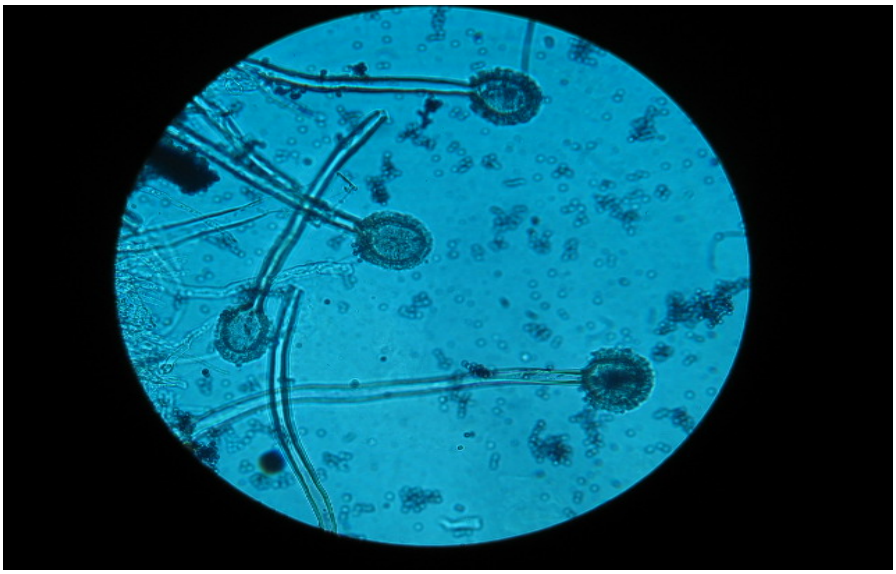

Fig. 3. *A. .awamori* NRRL 3112 grown in mineral media with crude (40X magnification)

The dry cell mass was found to be decreased in the mineral medium in comparison with that in the potato dextrose medium. The mycelia found in the two media were compared and it was found that the mycelia in the mineral medium were relatively thin when compared to that in the potato dextrose medium. However, during the stationary phase, cells were found clung as a collective mass on the hyphal elements, making the mycelia thickened. The specific growth rate in this medium was estimated to be 0.251 per day.

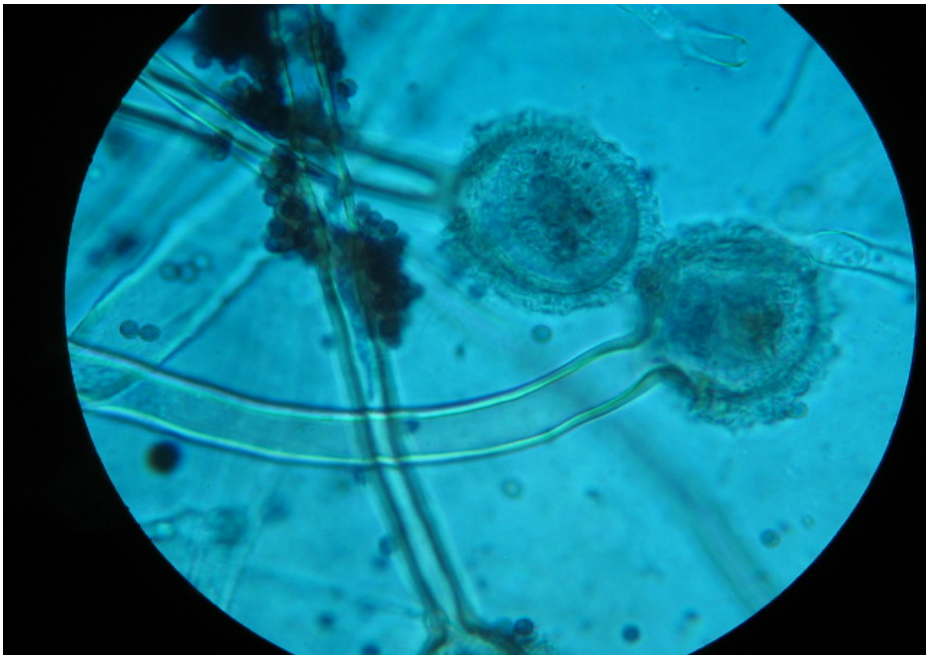

Fig 4. *A. awamori* NRRL 3112 grown in mineral media with crude in stationary phase

The initial pH, incubation temperature and period have been reported as significant process parameters which affect the yield of riboflavin utilizing fungal cultures (Kolonne et al., 1994; Lovallay et al., 1939). *A. awamori* NRRL 3112 was found to be capable of producing riboflavin under the experimental conditions employed. In order to gain more information concerning the influence of parameters which affected the vitamin formation under the present conditions of growth, a further set of experiments was carried out.

Effect of incubation period

The growth of *Aspergillus awamori* NRRL 3112 and its ability to produce riboflavin were estimated during a fermentation period which extended to 20 days. Riboflavin formation started on the fifth day and reached 143.75 µg/ml on the twentieth day. There after the formation was constant till the end of twentieth day. There was a decline in the vitamin formation after the twentieth day. The percentage of riboflavin dropped from 79.1 % on the twentieth day to 65.5% on the twenty fifth day. Riboflavin production occured from 10 to 20 days which was the stationary phase of the microorganism (Stahmann et.al, 2000). Sabry et. al (1989) also have made similar observations for riboflavin yields which were found to be maximum after the twenty day incubation period.

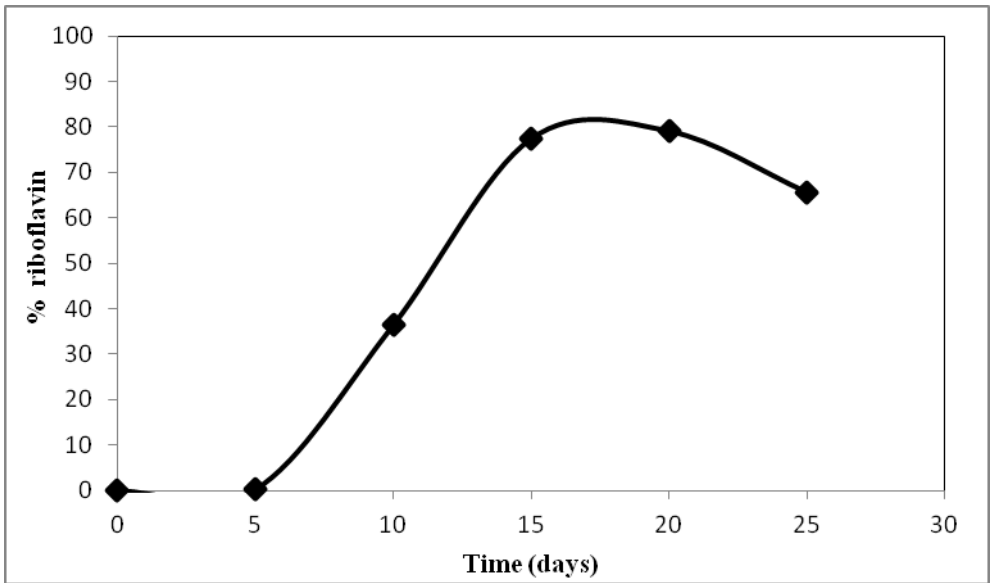

Fig.5. Effect of incubation period on yield of riboflavin from *A. awamori* NRRL 3112 grown in medium with crude oil

Effect of pH

Propagation and metabolic activity of any microorganism is highly affected by the pH. Adequate information is reported in the literature which indicates that the pH variations have a significant effect on the riboflavin production by microorganisms (Kolonne et al., 1994). pH values of 3.0, 5.0 and 6.4 were employed to determine their effect on riboflavin production. From the figure, riboflavin production was maximum at pH 5.0 followed by pH 6.4 and then pH 3.0. Literature reported earlier indicate that pH 5.6 was optimal for vitamin B_2 production by *Candida sp.* (Rodionova et.al, 1969) while pH 5.2 was optimal for *Pichia* sp. (Foster, 1962). However, pH 6.4 was recorded the best for the production of vitamin from hydrocarbons by *Pichia gulliermondii* (Nishio and Kamikubo, 1971). The specific growth rates at pH 3.0, 5.0 and 6.4 were found to be 0.119 per day, 0.536 per day and 0.251 per day respectively.

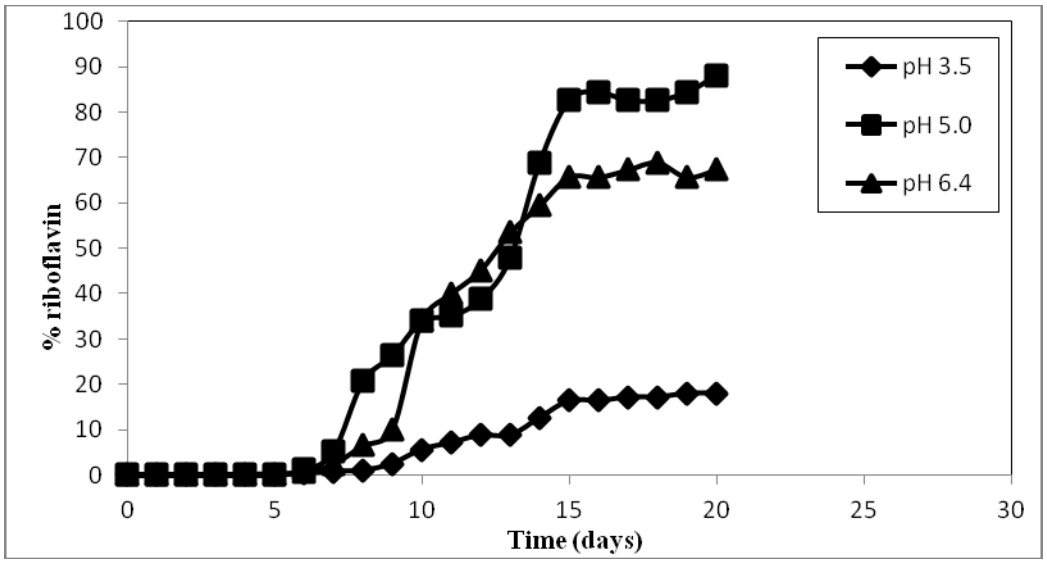

Fig.6. Effect of pH on yield of riboflavin from *A. awamori* NRRL 3112 grown in medium with crude oil

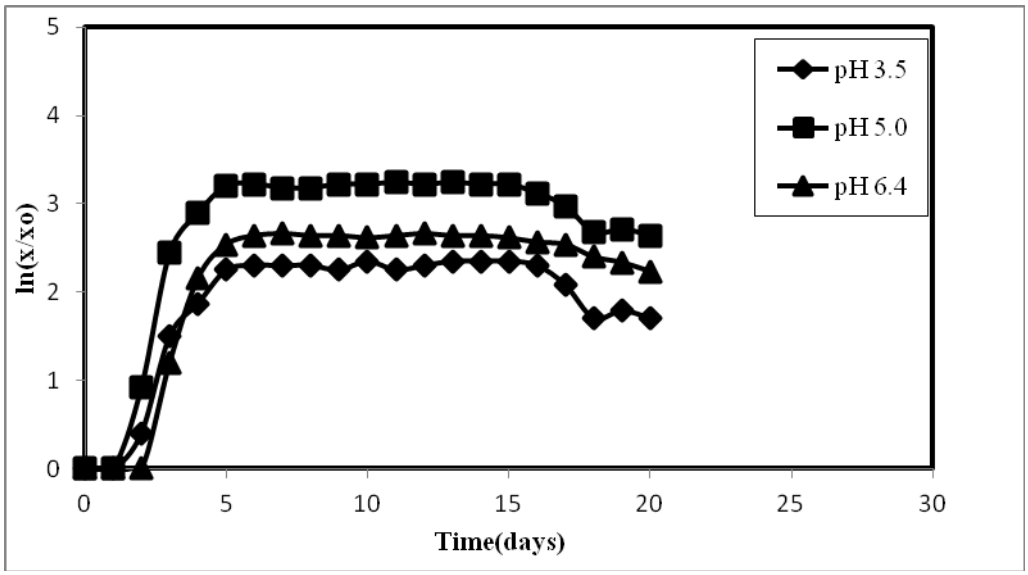

Fig.7. Effect of pH on growth of *A. awamori* NRRL 3112 grown in medium with crude oil

Effect of temperature

As the yield of riboflavin was high at pH 5.0, a temperature variation was tried over that range. Our observations indicate that there was a significant enhancement of the riboflavin production at 25^{o}C when compared to 30^{o}C. Significant growth was also observed at lower temperatures. The vitamin production was proportional to the growth rate of the microorganism in the stationary phase, reaching its maximum at 25^{o}C. Thus a temperature of 25^{o}C appeared to be the best suited for obtaining higher riboflavin yields at pH of 5.0. Temperatures above 30^{o}C were not considered as earlier reports indicate no measurable growth or riboflavin production at higher temperatures (Sabry et al., 1989; Baruah 1978)

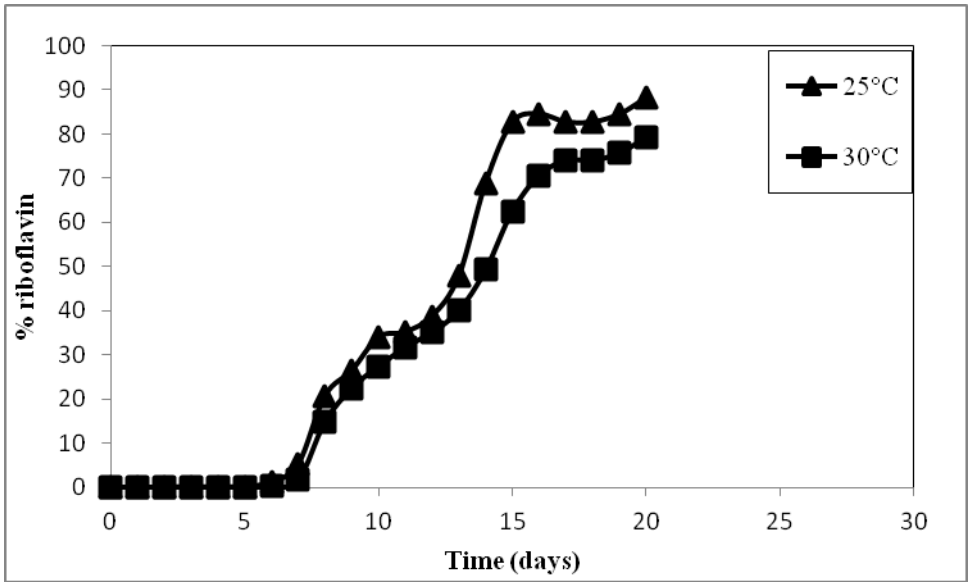

Fig.8. Effect of temperature on yield of riboflavin from *A. awamori* NRRL 3112 grown in medium with crude oil

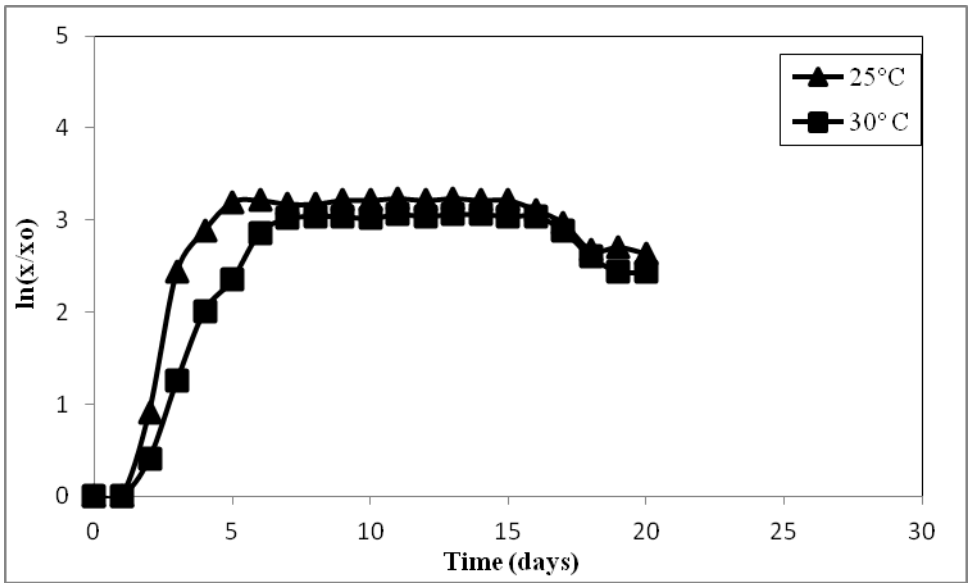

Fig.9. Effect of temperature on growth of *A. awamori* NRRL 3112 grown in medium with crude oil

Conclusion

The potentiality of the fungus, *Aspergillus awamori* NRRL 3112 to produce riboflavin by utilizing hydrocarbons was tested. It was seen that, the fungus has a good growth rate of 0.312 per day in potato dextrose medium and 0.251 per day in mineral media with crude. The fungi exhibited denser growth when it was acclimatized. When the incubation period was varied maximum riboflavin concentration of 143.75 µg/ml was obtained on the twentieth day. When a range of pH including 3.0, 5.0 and 6.4 was employed, maximum growth and maximum riboflavin percentage of 88% was obtained at pH 5.0. On changing the temperature, it was found that lower temperature gave a higher yield of riboflavin. Thus, based on the results obtained from the conducted experiments, it can be concluded that, the fungal strain *Aspergillus awamor*i NRRL 3112 can be considered as a potential microorganism to produce riboflavin in significant quantities using the fermentative route. Further, investigations on the effect of a wider range of temperature, pH on the riboflavin production, the effect on different hydrocarbon sources on the yield of riboflavin are in progress.

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