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# Optimization of Fermentative Production of Shikimic Acid by *Pseudomonas putida*

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## ABSTRACT:

Shikimic acid, one of the chiral compounds, is widely used as key precursor for synthesis of Tamiflu, the only drug against avian flu caused by H1N1 virus. It is also highly demanded by pharmaceutical and cosmetic industries. Due to its scarcity, currently scientists are making attempts to explore fermentative route of production of shikimic acid as alternative to present extractive method. Although numbers of genetically modified micro-organisms are being used for this purpose, very little work has been reported using wild microbial strains. *Pseudomonas putida* possess potential capacity of production of shikimic acid. The present work aimed at optimization of fermentative production of shikimic acid using *P.putida*. To optimize the process different physiological and nutritional parameters were checked, which include : effect of addition of different carbon sources, different carbon concentration, addition of nitrogen source ,temperature , pH, addition of glyphosate, aeration, agitation. In our study, P.putida initially produced  g/L of shikimic acid. However when process was employed under optimized conditions g/L of shikimic acid was produced.

**Keyword:** Shikimic acid, avian flu, scarcity, fermentation, *Pseudomonas putida*, Optimization.

## I. INTRODUCTION

Shikimic acid (3,4,5-trihydroxy-1-cyclohexene-1-carboxilic acid), a white crystalline organic compound, is an important intermediate of 'shikimate pathway' in all plants and microbes [11]. It acts as vital precursor in synthesis of aromatic amino acids, tyrosine, tryptophan and phenylalanine and it is also responsible for synthesis of other compounds (such as phenolics, alkaloids and phenyl propanoids) required for growth of plants [14]. It is a versatile enantiomer as its structure contains highly functionalized six-carbon ring with three chiral carbons and a carboxylic acid group [15].

Due to its chiral nature shikimic acid is widely used as key precursor for synthesis of Tamiflu, the only drug against avian flu caused by the

H5N1 virus. Shikimic acid is converted to a diethyl ketal intermediate which is then reduced in two steps to an epoxide that is finally converted to Tamiflu (Oseltamivir). It is also used in pharmaceutical and cosmetic industries. Shikimic acid can also be converted into compounds that play important role in inhibition of cell proliferation and due to this they can be used as anti-cancer chemotherapeutic agent [5].

It is also used as additive to food and feed and injectables [9].

Shikimic acid pathway is widely distributed in plants and micro-organisms. Benzene ring which is the basic element of all the aromatic amino acids forms in plant and micro-organisms by Shikimic acid pathway [9]. Starting components of Shikimic acid pathway are phosphoenol pyruvate resulting from glycolytic degradation of glucose and eythrose-4-phosphate, which is oxidative product of pentose phosphate pathway. Condensation of both of these compounds is inducing step that leads to the formation of Shikimic acid.

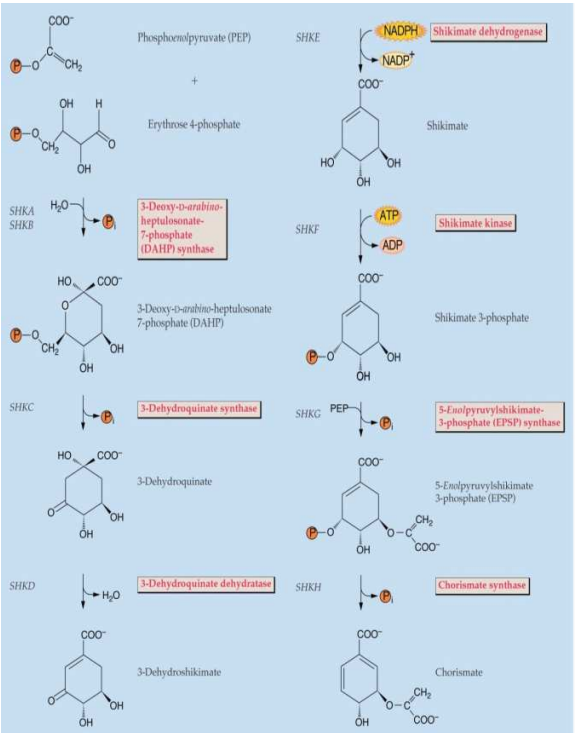


**Fig.1.**Shikiic acid pathway

Further transformations of Shikimic acid are responsible for synthesis of another important intermediate called chorismic acid at which shikimate pathway divides. The first direction gives rise to second form of L-phenylalanine and L-tyrosine. The successive transformation involved in second branch results in production of phenolic compounds in plants [13].

The widely used plant source of shikimic acid is fruits of a Chinese plant star anise (*Illicium vernum*) a woody shrub. However the tree is not cultivated easily, attaining its first seed bearing stage after 6 years of growth, it is unlikely that the growing market demand would be met by the *l .verum* source alone. So the efforts are made to explore new sources of shikimic acid. Being inexpensive, renewable resource, fermentative production of shikimic acid using microbes is one of the most attractive route of shikimic acid production.

Many scientists have made attempt to produce shikimic acid using metabolically engineered strains of micro-organisms. The micro organisms reported to be engineered for this purpose up till now are *E.coli*, *Citobacter freundii*, *Bacillus amyloliquefacians and Aerobacter aerogenes* [2].

In early studies, ability of '*Pseudomonas putida*' to undergo shikimic acid pathway was conformed. The current study deals with obtaining maximum yield of shikimic acid by fermentative process using *Pseudomonas putida.*

## II. MATERIALS AND METHODS

### 2.1 Materials

All chemicals and reagents used in this study were of analytical grade obtained from commercial sources.

### 2.2 Media and culture conditions

Here, media used for production of shikimic acid was M9 minimal media which contained following components (gram per liter of de-ionised water) : Carbon source, 50 g ; $Na_2HPO_4$ , 30g ; $KH_2PO_4$, 15g; NaCl , 2.5; $NH_4Cl$, 5g; 2ml of $MgSO_4$. After that volume of medium was

adjusted to1000 ml with de-ionized water and then it was adjusted to pH 7.3 with 1N NaOH or 1N HCl. Then the medium was autoclaved at 121$^{o}$C for 15 min. The seed medium was of same composition. During study of effect of nutritional parameters on shikimic acid production addition of specific media supplements like nitrogen source, amino acids, glyphosate etc. was done at that particular instant as per the requirement.

**2.3 Microbial Strain Used**

DF-SI- 1 strain of Pseudomonas putida was used for this study purpose. The culture of P.putida was maintained by preparing its slants on M9 minimal and nutrient medium and storing them in deep fridge.

**2.4 Preparation of inoculum**

Autoclaved 5ml M9 minimal media was then into clean sterile test-tube. After that with help of Nicrome wireloop one loop full of preserved culture of *P.putida* was added to medium and mixed uniformly. Then it was kept for incubation at room temperature for 24 hrs. 4. After completion of incubation growth of micro-organism was confirmed by observing turbidity of medium and then this was used as inoculum to carry out fermentation.

**2.5 Production and optimization**

**2.5.1 General scheme of production**

The production was carried out on flask scale. To carry put fermentation, sterile 100ml of M9 minimal medium was added to 250ml conical flask to which 1ml of 24hr old culture of P.putida was added. Then the flask was kept for fermentation on rotary shaker at room temperature until the stationary phase was reached. During fermentation time to time sampling was done and analysis of shikimic acid was done by following standard protocol of assay for shikimic acid production.

**2.5.2 Assay for shikimic acid detection**

When samples were taken out for analysis, at first they were centrifuged at 10,000rpm for 10 min on spinwin. Then pellet was discarded and supernatant was collected. 3ml of this supernatant was collected in a clean, dray test-tube to which 0.5ml of 1% periodic solution was added and mixed uniformly. Then this mixture was kept for incubation at room temperature for 3 hrs. After completion of incubation period 0.5 ml of 1N NaOH was added to that solution. And immediately after that 0.1 ml of 0.1N Glycine was added and absorbance was taken at 380nm on UV-spectrophotometer.

**2.5.3 Optimization studies**

Sometime changes in culture conditions may result in considerable increase in product yeild; hence during optimization study of effect of some nutritional and physical parameters was done.

**2.5.3.1 Media optimization**

**Effect of carbon source**

To select best carbon source that provides maximum shikimic acid yeild, effect of various carbon sources on shikimic acid production was studied. This study was done into two steps:

In first step, 6 different carbon sources i.e Glucose, Glycerol, Tri-Sodium citrate, Sucrose, Fructose, Maltose at their final concentration of 50 g/L were added to M9 minimal medium and fermentation was carried out by following standard protocol.

In second step, shikimic acid production was tested at different concentrations of carbon source that was selected as best carbon source in first step. The Concentration range selected for this was 2% to 20%.

**Effect of addition of nitrogen source**

Earlier it was reported that addition of nitrogen source resulted in increase in product yeild. Hence this parameter was studied. Yeast extract was added as nitrogen source to medium. Two concentrations of yeast extract i.e. 5 g/L and 10 g/L were used. Fermentation was carried out until

the stationary phase was reached. Sampling and analysis was carried out time to time.

**Effect of addition of aromatic amino acids**

Aromatic amino acids i.e Tyrosine, Tryptophan and Phenylalanine are the end product of the shikimic acid pathway. To check whether the addition of these amino acids at initial stage of fermentation causes any alteration in shikimic acid production, 50mg/L of each amino acid was added to the medium and fermentation sample were checked for shikimic acid production.

**2.5.3.2 Optimization of physical parameters**

**Effect of temperature**

Temperature is one of the most vital factors for fermentative processes that may cause considerable fluctuations in microbial growth and growth related product yeild, hence in that regards, shikimic acid production was studied on temperature range 25-45$^0$C.To maintain these temperature shaking incubator was used.

**Effect of pH**

Along with temperature, pH also causes alteration in product yeild. Therefore, shikimic acid production was carried out at 4 different pH 2,4,7,9.

**Effect of addition of Glyphosate**

Glyphosate, widely used herbicide, has an inhibitory effect on shikimic acid pathway. Earlier it was reported that glyphosate inhibits incorporation of shikimate into all three aromatic acids. Due to which it accumulates shikimic acid causing increase in production of shikimic acid. Hence, here we checked effect of addition of glyphosate to media. This study was carried out in two parts-

**I) Effect of different concentration of glyphosate.**

As shikimic acid is secondary metabolite, it mainly accumulates in stationary phase. Hence for this study; 10ml of different concentration of glyphosate was added to 100 ml of M9 minimal medium after 6$^{th}$ day of fermentation. Concentration range of glyphosate selected for study was 5mM to 20mM. Sampling and assay were carried out as before.

**II) Effect of addition of glyphosate at different time interval.**

The previous study gave concentration of glyphosate that shown maximum accumulation of shikimic acid on addition. By using that data, further study to know, during fermentation at which stage addition o glyphosate gives increased level of yeild, was carried out. For that, 10 ml of concentration that had shown maximum yeild in first step of this study was added to fermentation broth at different time intervals. The time intervals selected here were after 2$^{nd}$ day, 4$^{th}$ day and 6th day. Then the sampling and assay to detect shikimic acid was carried out as per the standard protocol.

**Final runs on flask scale**

After obtaining all optimum parameter that gave maximum amount of shikimic acid, by using these conditions a final run on flask scale was carried out before validating the process.

**Validation of process on 1-L lab scale fermenter**

Final validation of process was carried out on 1-L lab scale fermenter by using all the optimum conditions. 500 ml of M9 minimal media along with nitrogen source, aromatic amino acids was inoculated with 5ml of 24hrs old culture of *P.putida*. As P.putida is an aerobic micro-organism, during fermentation along with agitation, aeration also maintained to check its effect on production. Agitation was maintained by impellers provided in fermentation vessel. Agitation of 100 rpm was maintained. Aeration was supplied by using air compressor. Air flow rate was maintained as 0.5 $m^3$/L. Temperature was maintained by cooling jacket provided around the fermentation vessel. Desire pH, DO was maintained by pH and DO probe available in fermentation assembly. Fermentation was carried out until stationary phase was reached. During fermentation sampling was done at particular time interval at sampling port. Assay to conform

presence of shikimic acid fermentation was carried out by standards protocol.

**Fig. 2(a)** Lab scale fermenter

**Fig. 2(b)** Lab scale fermenter

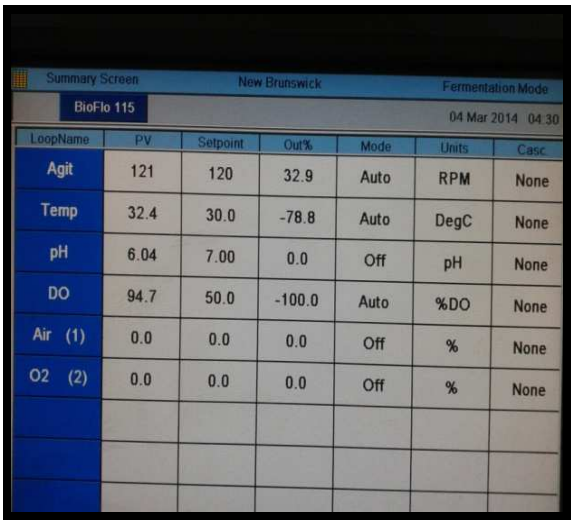


**Fig. 2(c)** digital screen touch display

## III. RESULTS AND DISCUSSIONS

**Effect of different carbon source on shikimic acid concentration**

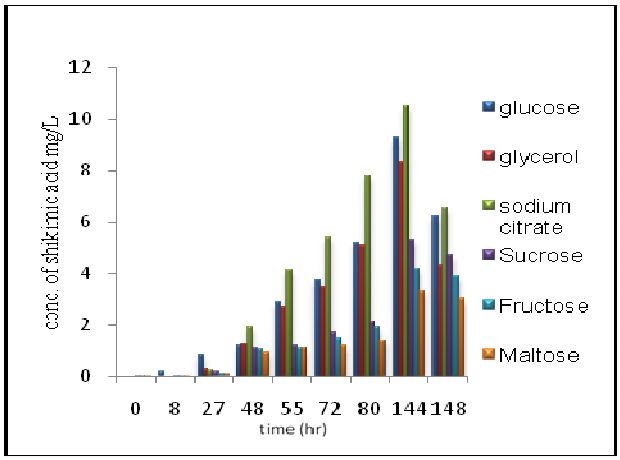


**Fig. 3(a)** Effect of different carbon source

As shown in results, addition of 5% glucose, sodium citrate and glycerol enhanced the production of shikimic acid. However other sugars such as maltose, sucrose, Fructose didn't yeild in good amount of shikimic acid. Under these conditions maximum 10.4 mg/l of shikimic acid was produced in late exponential phase. In order to know the best carbon source among the three source that shown maximum yeild, again they were checked for shikimic acid production. Here sodium citrate gave the maximum shikimic acid yeild than glycerol and glucose.

In terms of shikimic acid production, cost for industrial application and less chances of contamination, sodium citrate was selected as most suitable carbon source.

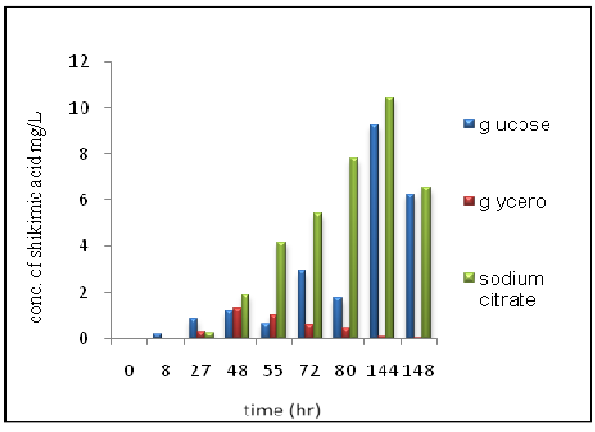


**Fig. 3(b)** Effect of different carbon source

**Effect of different concentration of sodium citrate**

After that, production of shikimic acid at different concentration of sodium citrate was studied. Among the different concentrations selected, 2% sodium citrate gave maximum yeild. On the other hand there was absolutely no growth of microbe was seen in case of 15% and 20% and hence they were discarded. This was may be because of substrate inhibition of microbial growth. As the substrate concentration increased the growth of microbe was inhibitated.

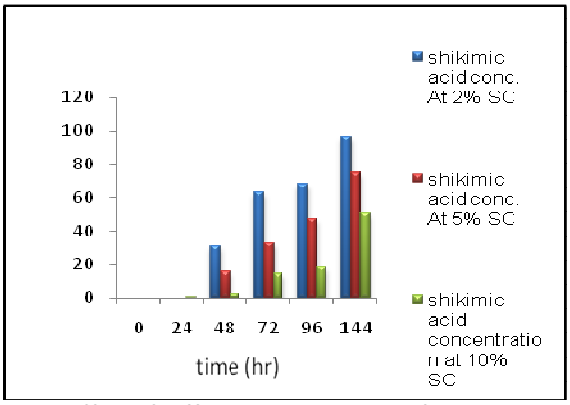


**Fig.4.**Effect of different concentration of sodium citrate

**Effect of addition of Nitrogen source**

When yeast extract was added to medium as nitrogen source with its two concentration i.e 5mg/l and 10mg/l then it was observed that 10g/L of yeast extract shown considerable increase in shikimic acid with 141 mg/L. However 5g/L of yeast extract gave less shikimic acid production. hence 10g/L of yeast extract was selected as most suitable for shikimic acid production.

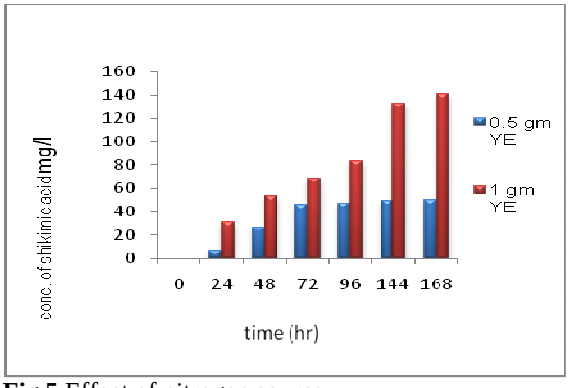


**Fig 5**.Effect of nitrogen source

**Effect of addition of aromatic amino acid**

When 50mg/L of each end product of the shikimic acid pathway i.e. aromatic amino acids i.e tyrosin, tryptophan and phenylalanine was added to M9 minimal medium and fermentation was carried out following standard process, tremendous increase in shikimic acid production was observed with about 200 mg/L of shikimic acid yeild.

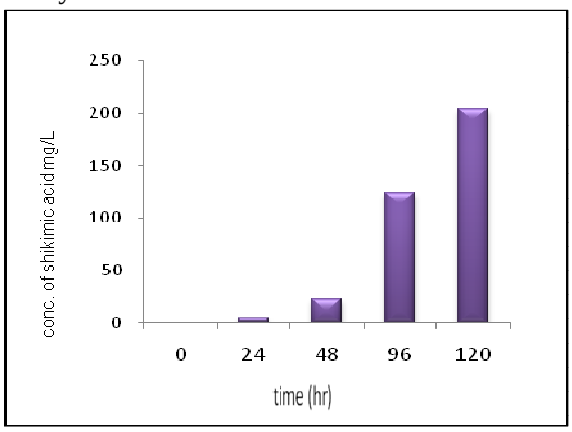


**Fig.6.**effect of addition of amino acids

**Effect of temperature**

Among the different temperature such as 25°C, 30°C, 35°C and 45°C tried, maximum shikimic acid production was observed at 30°C with optimal microbial growth. However at 25°C and 35°C, the growth and production of shikimic acid was less. At 45°C absolutely no growth was seen hence that set of fermentation broth was discarded. The most probable reason could be that the present strain being a soil and natural environment habitat microbe that grows very well at normal temperature range of 30°C to 37°C and produces maximum shikimic acid at this temperature**.**

**Effect of pH**

As shown in result (fig.9) that though shikimic acid was produced in the wide range of 4-9.The increased production of 56.25 mg/L shikimic acid was observed at pH 4.Thus, pH 4 was selected as optimum pH for shikimic acid production

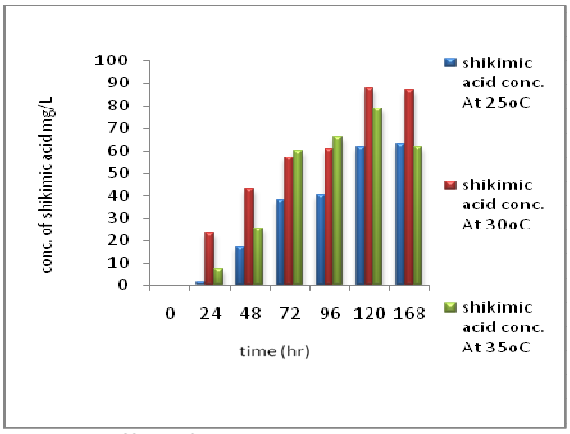


**Fig.7** Effect of temperature on shikimic acid

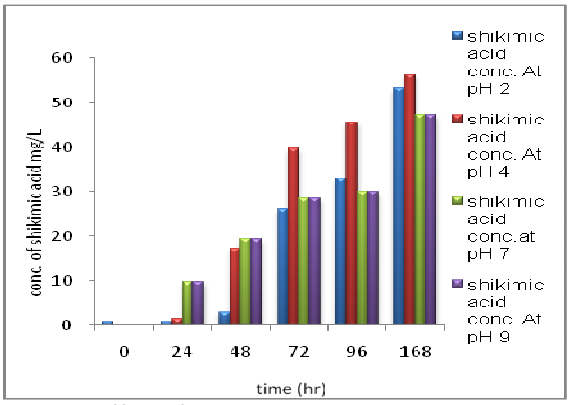


**Fig.8**.Effect of pH on shikimic acid production

## Effect of addition of glyphosate

When for different concentration of glyphosate ranging from 5mM-20mM, production of shikimic acid was checked, it was observed that though the shikimic acid was produced on wide range of glyphosate concentration, 20mM of glyphosate gave maximum shikimic acid accumulation and hence increased product yeild.

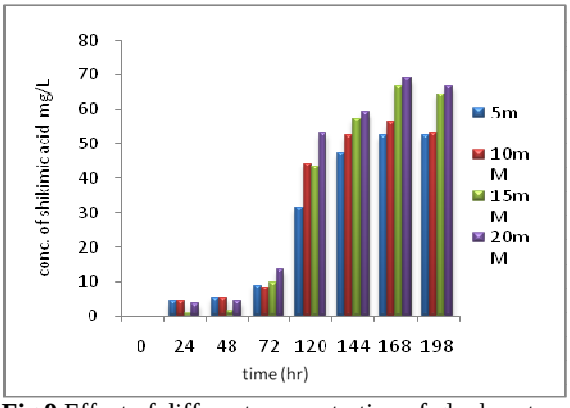


**Fig.9** Effect of different concentration of glyphosate on shikimic acid production

After came to know that the 20mM of glyphosate gives maximum shikimic acid production, time at which glyphosate should be added to medium to enhance the production of shikimic acid was determined. Among the time intervals selected, addition of glyphosate after 6$^{th}$ day of fermentation gave maximum shikimic acid production. Shikimic acid yeild obtained on addition of glyphosate after 2$^{nd}$ day and 4$^{th}$ day was less significant. Hence addition of 20mMof glyphosate after 6$^{th}$ day of fermentation was selected as suitable condition for shikimic acid production (Fig.11).

## Final flask scale run

When by using all the optimum parameters selected final flask scale was carried out , it was observed that there was considerable increase shikimic acid production with about 163 mg/L of shikimic acid yeild.

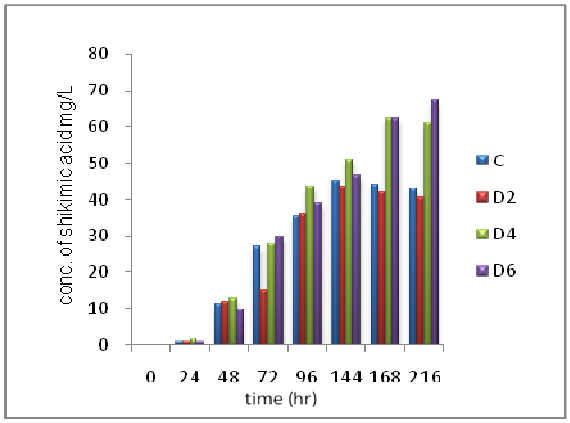


**Fig.10**.Effect of glyphosate on shikimic acid production at different time intervals

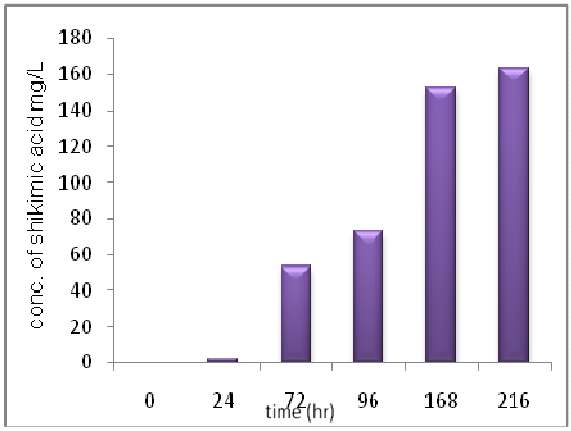


**Fig.11** Final flask scale run

**Process validation on 1-L fermenter and effect of aeration on shikimic acid Production**

After validating the process on 1-L lab scale fermenter under all optimized conditions and with supply of air having flow rate 0.5 $m^3$/L, tremendous raise in shikimic acid production was seen (370 mg/L) .About 3-fold increase in product yeild was observed (fig.12).This would be because, as *P.putida* is an aerobic micro-organism.

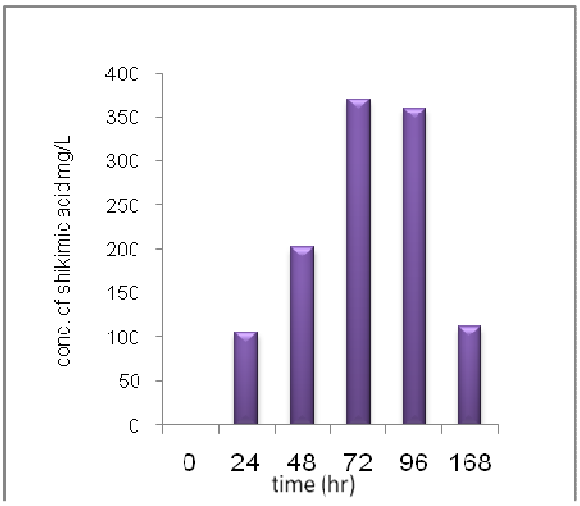


**Fig.12** Validation of process on 1-L lab scale fermenter

## IV. CONCLUSION

In this study, production shikimic acid by using Pseudomonas putida was carried out. Process of shikimic acid production was optimized by determining effect of various nutritional and physical parameters. It is of great significance that the process engineering resulted in 3-fold enhancements in shikimic acid production with increase in product yeild from 161 to 370 mg/L. To date, there have been no reports of wild-type strain of *P.putida* producing shikimic acid under these optimized conditions. Therefore, the study suggested that more optimization studies can further increase the yield of shikimic acid by *P.putida*. Further, the results obtained in the present research indicate that the *P.putida* could be a promising agent for commercial production of shikimic acid and its application in pharmaceutical industry. Moreover, these data would lay the foundation for future research on the utilization of this strain to achieve higher shikimic acid production.

## V. FUTURE ASPECTS

Recently, various micro-organisms like *E.coli, Citobacter freundii, Bacillus amyloliquefacians and Aerobacter aerogenes* have been genetically transformed to obtain higher amount of shikimic acid by means of fermentation (Bochkov et al., 2011).

In future, genetic manipulation of *P.putida* would explore new alternative to obtain high shikimic acid production on industrial scale application.

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