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Study on growth and ascomycin production by *Streptomyces hygroscopicus* subsp. *Ascomyceticus*

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ABSTRACT

The objective of this study undertaken was to acquire maximum information by the shake flask studies for Ascomycin production with *Streptomyces hygroscopicus* subsp. a*scomyceticus* ATCC 14891. The morphological observation of culture on plate media was carried out visually and under stereo microscope. The colonies obtained were granular and attained grayish black color on maturity. The microscopic examination of mycelia was performed at 20× and 100× (oil immersion) magnification. To improve the Ascomycin production in shake flask studies, two different approaches were evaluated. In the first set of experiments, "Two stage Fermentation" approach was adopted wherein; matured lab inoculum was transferred directly in different production media. The second approach consisted of set of experiments with "Three stage Fermentation" which involved transfer of grown lab inoculum into seed media and grown seed culture into production media. The age of seed culture was investigated for the betterment of the yield. The maximum concentration of ascomycin obtained was 0.5 mg/gm in the second approach on selected P8 media. Media selection was done on the basis of carbon source, nitrogen source, trace salts, buffer to maintain pH throughout the process and precursor as supplement. Simultaneously, microscopic observation was captured in the present study. Thus it could be concluded that "Three stage Fermentation" on selected media was the best option for scale up of ascomycin.

1. Introduction

Classical fermentation has been widely used for the production of various economically viable and sustainable products of biological importance. Fermentation processes are used for centuries to produce sustainable pharmaceutical products and to meet the market requirements in a cost effective manner.

Various microorganisms have been reported to produce primary and secondary metabolites. Actinomycetes, a class of beneficial bacteria play a major role in producing different kinds of secondary metabolites which are biological active substances. Interestingly *Streptomyces*, the largest genus of Actinobacteria harbor the potential to produce a multitude of bioactive secondary metabolites, including, but not limited to the important therapeutic categories of antibiotics, immunosuppressant, antifungal, and anti-cancer compounds (Singh et al., 2017).

In order to maximize the metabolite output, several tools and techniques have been tried and successfully implemented in various biotechnological processes, like production of primary or secondary metabolites, biotransformation etc. (Dubey et al., 2008; 2011; Singh et al., 2009; Rajeswari et al., 2014). Medium optimization by shake flask studies is one of the most important critical steps that are carried out before any scale up of fermentation process. So, for designing an efficacious production media, fermentation parameters such as pH, temperature, agitation speed, and dissolved oxygen as well as the appropriate medium components must be identified and optimized. An increase in yield makes the process cost competitive which is a dire necessity in the changing market scenario. (Singh et al., 2017).

Ascomycin, a well-known immunosuppressant and antifungal, is used for the treatment of autoimmune diseases and skin diseases, and to prevent organ rejection after transplant. Apart from active pharmaceutical ingredient, Ascomycin is useful as a key starting material for the synthesis of Pimecrolimus (Kumar et al., 2007). Ascomycin is produced industrially by microbial fermentation through *Streptomyces hygroscopicus* subsp. *ascomyceticus.*

Tadashi et al. (1966) reported an antifungal agent identified as ascomycin which is prepared by the process which comprises cultivating a strain of *Streptomyces hygroscopicus* var.*ascomyceticus* under submerged aerobic condition.

The isolation of active metabolite from microorganism is relatively

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safe as compared to chemical synthesis of antimicrobial agents. Due to important medical value and market prospects, it will be of great significance to improve fermentation titer of ascomycin or FK 520 by strain evolution and fermentation optimization (Hai-shan et al., 2012).

Available literature on Ascomycin cited very few research articles on the media optimization and culture characteristics. Hence the focus of this study was optimization of media components by shake flask studies. So, the present study was envisaged to maximize the growth of *Streptomyces hygroscopicus* subsp. *ascomyceticus* ATCC 14891 and thereby increase ascomycin production in scale up studies.

2. Materials and methods

2.1. Microorganism and maintenance

In this study, *Streptomyces hygroscopicus* subsp. *ascomyceticus,* ATCC 14891 was used for the production of ascomycin by submerged fermentation. The culture was maintained on growth medium (YMA) containing yeast extract 4.0 gm/L, malt extract 10 gm/L, glucose 4 gm/L and agar 15 gm/L with pH 6.5–7.0. The culture was incubated at 28 °C for 10–14 days. Individual colonies were transferred to slants containing same media and after maturation stored at 4 °C.

2.2. Characteristics of Streptomyces hygroscopicus subsp. Ascomyceticus, ATCC 14891

Morphological characterization in term of growth pattern and microscopy was done as per Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

2.3. Shake flask process

2.3.1. Development of lab inoculum

Grown YMA slant was harvested with 5 ml of normal saline and 0.5 ml of culture suspension was inoculated in 30 ml Lab medium (30 ml media in 250 ml conical flask) and incubated for 48 ± 8 h at 28 °C and 200 rpm (Table 1).

2.3.2. Development of seed culture

S1 and S2 seed media were prepared as per the composition given in Table 2. 3% of grown lab inoculum was transferred into 30 ml S1 and S2 seed media in 250 ml flask respectively. These flasks were incubated at 28 °C at 240 rpm up to 36 ± 12 h.

2.3.3. Transfer of grown lab inoculum and seed culture into production media

Different media were selected from the available literature and formulated as per nutritional value and tested for the production of ascomycin. A microbe used for fermentation, grow in specifically designed growth medium which supplies nutrients required by the organism. In a fermetation process, composition of culture medium plays a major role and will determine to a great extent the level of end product. A variety of media invariably contain a carbon source, a nitrogen source, water, salts and micronutrients.

Two seed media (S1 and S2) and eight different production media were selected to obtain the higher yield. Refer Tables 2 and 3 for the media composition for seed medium and production medium respectively. The shake flask fermentation media were formulated in 250 ml conical flask containing 30 ml media. In the present study, two approaches (two sets of experiments) were implemented for ascomycin production in shake flask. In the first set of experiments contained two stages of fermentation and three stage of fermentation was applied to the second set of experiments.

Two stage fermentation: 10% grown lab was transferred to each production media flask (P1 to P8), and incubated at 28 °C and 200 rpm for 11 days.

Three stage fermentation: 3% of grown lab inoculum was transferred into both seed media (S1 and S2) and further 10% grown seed culture was transferred to production flask. The flasks were incubated

Table 1
Lab medium composition.

S. No.	Component	Quantity (g/L) [a]
1	Yeast extract	4.0
2	Malt extract	10
3	Dextrose	4

[a] The pH of the medium was adjusted to get the desired pH of 6.60–6.90 after sterilization.

Table 2
Seed media composition.

S. No.	Component (g/L)	S1	S2
1	Dextrose	1.0	8
2	Dextrin White	10.0	
3	Milk	2.0	
4	Cotton seed meal	2.5	
5	Tryptone	5.0	
6	$MgSO_4$	0.05	
7	$FeSO_4$	0.025	
8	NaCl	0.5	1.0
9	$CaCl_2$	0.02	
10	$ZnSO_4$	0.01	
11	$MnSO_4$	0.005	
12	Phosphate Buffer pH 6.8	2 ml	
13	Corn starch		10.0
14	Soya flour		20.0
15	Inactive dry yeast		1.0
16	Yeast Extract		0.5
	pH adjusted to	7.2	7.2

Table 3
Production media composition.

S. No.	Component (g/L)	P1	P2	P3	P4	P5	P6	P7	P8
1	Dextrose	5		5					
2	Soluble starch	5	2.50		20	24			
3	Beef extract	5							
4	Bacteriological peptone	10			10	5			
5	Sodium Chloride	5							
6	Soya flour		20	10	5		10	10	30
7	Ebios (active yeast)		0.50						
8	Magnesium Chloride		0.01						
9	Copper Sulphate		0.01						
10	Zinc Sulphate		0.03						
11	Calcium Carbonate		3.50	1.5	1	1	1.5	1.5	2
12	Dextrin white			90	40	40	140	30	
13	Soya peptone			10			10	10	
14	Glycerol			10			10	10	15
15	L-Lysine			2.5					
16	Di-Potassium hydrogen phosphate			1			0.8	0.8	
17	Polyethylene glycol			1			12.5	12.5	
18	Corn steep powder				10	2			
19	Potassium dihydrogen phosphate				1	1			
20	Ammonium sulphate				1	1.5			
21	Magnesium Sulphate				1	1			
22	Yeast extract powder					7			
23	Shikimic acid					1.5			
24	Soya oil					2.5			
25	Cotton seed meal						14	10	
26	Corn starch								30
27	Maltodextrin								10
28	Maltose								25
29	Inactive dry yeast								5
	pH	7.0	7.0	7.0	7.2	7.2	7.2	7.2	7.0

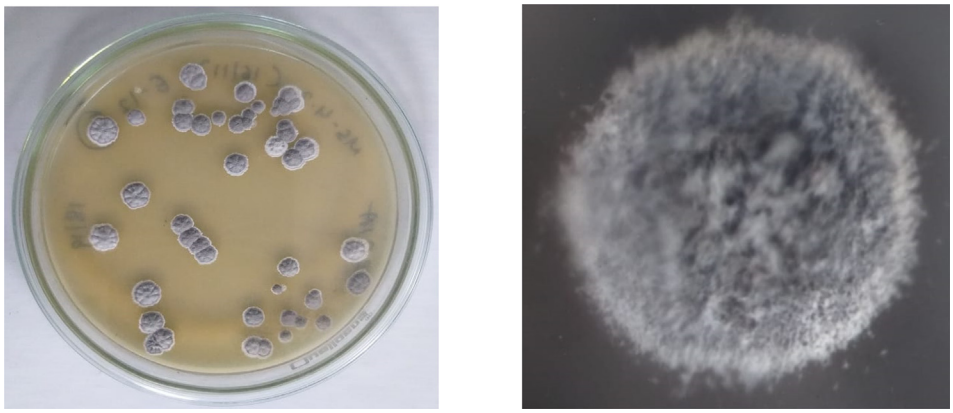

Fig. 1. Visual and Stereo (single colony) image of culture.

at 28 °C and 200 rpm for 11 days.

2.3.4. Physical parameter determination

The physical process parameters like pH, %PMV and microscopic characteristics were estimated at different intervals (from day 1 to day 11 at every 24 h interval).

2.3.4.1. pH of culture broth. pH meter was calibrated with neutral (7.0), acidic (4.01) and alkaline (10.01) buffer. pH electrode was rinsed with demineralized water and wiped it carefully with a tissue paper. Immerse the electrode into the sample and note down the stabilized pH value.

2.3.4.2. Packed mycelial volume (PMV). The PMV of the sample was determined by putting 10 ml broth sample in 15 ml graduated tube and centrifuge at 5000 rpm for 5 min. The PMV was the pellet volume expressed as a percentage (%) of measured total volume of the tube.

$$\text{PMV\%} = \frac{\text{Precipitate volume(ml)}}{\text{Total Volume}} \times 100$$

2.3.4.3. Microscopy. A smear was prepared by taking loop-full of culture suspension that is to be stained. Heat fixed the smear so that culture gets firmly mounted onto the slide. Gram staining was done and observed under the microscope.

2.4. Determination of age of seed culture

Seed flaks were inoculated with 3% grown lab inoculum and incubated at 28 °C on a rotary shaker at 200 rpm for different time interval (36 h, 40 h, 44 h and 48 h). At every interval, seed culture was transferred in production flask and run the experiment for 11 days.

2.5. Analysis of ascomycin in broth samples through HPLC

Concentration of ascomycin produced in the culture broth was determined by HPLC (Water, pump-alliance (2695), auto sample-alliance (2695); detector –UV (2489) with empower software) method. Acetonitrile was used to extract the fermentation broth. 5 gm sample was weighed and dissolved in acetonitrile and filtered. A mobile phase of 0.01% trifluoroacetic acid and acetonitrile in 1:1 ratio was used in isocratic mode. Ascentis Express C18, 100 × 4.6 mm, 2.6 µ column was used to estimate ascomycin concentration. The flow rate was set at 1.0 ml/min. The resulting solution (20 µl) was injected into the HPLC for the estimation of ascomycin. Concentration of Ascomycin present in the fermentation broth was calculated by comparison of peak areas with that of ascomycin standard.

2.5.1. Preparation of solutions

A) Standard Solution: Weighed 10 mg ascomycin standard in 50 ml volumetric flask and made up to volume with acetonitrile.

B) Sample Solution: Weighed about 5 gm of broth sample in 25 ml volumetric flask, add about 10 ml of acetonitrile sonicate, make up the volume with acetonitrile.

3. Results

*3.1. **Visual and Stereo observation of** Streptomyces hygroscopicus subsp. Ascomyceticus ATCC 14891*

The colonies were circular in shape with irregular margin. They were moderate in size, white in color which turned grayish black on maturity and had elevated surface. They attained 8–10 mm diameter on maturity. The colonies were rugose as they had deep furrows radiating from the center. The appearances of the colonies were granular due to the presence of spores and possessed many radial folds and concentric rings. Refer Fig. 1 for isolated colonies of *Streptomyces hygroscopicus* subsp. *ascomyceticus*.

3.2. Microscopic observation

Streptomyces ascomyceticus is a Gram positive, aerobic, filamentous bacteria which produces well developed vegetative hyphae with branches and forms interwoven network of mycelia. The mycelia and aerial hyphae that arise from them are non motile, mobility is achieved by dispersion of spores. The branched filaments develop with radial symmetry are located in the central part of the network. The central part gradually undergoes various degrees of fragmentation with the age. Refer Fig. 2 for microscopic images.

3.3. Shake flask study

3.3.1. Development of lab inoculum

In lab media, the culture was enriched with required nutrients. To increase the purity of culture and minimize the toxicity of the medium, cells were nourished in lab media. The pH and %PMV were determined to know the concentration/volume of mycelia in medium. Matured lab inoculum at the age of 44 h with pH 6.9 and PMV% 6 was transferred in production as well as seed media. Growth profiling of lab inoculum was performed to determine pH, %PMV and age. As shown in Fig. 3, the parameters were recorded from 12 h to 42 h at every 6 h interval and then 2 h interval from 42 to 48 h.

3.3.2. Development of seed culture

Two different seed media were evaluated on the basis of literature. S1 and S2 seed media were inoculated with 44 h grown lab culture.

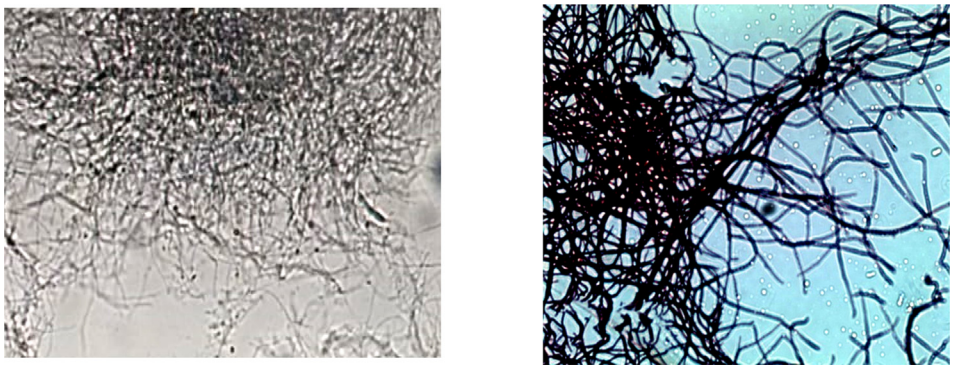

Fig. 2. Microscopic observation of culture at 20X and 100X.

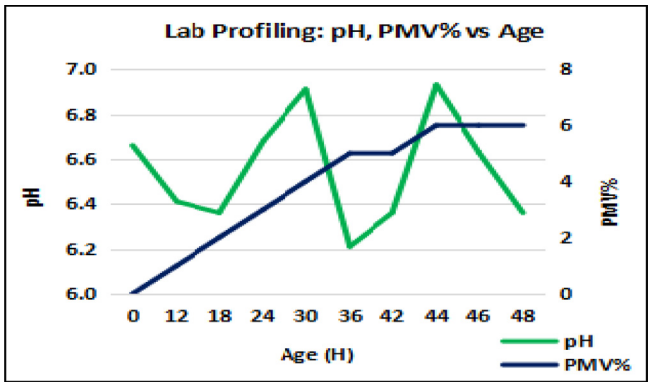

Fig. 3. Lab inoculum profiling.

Transferring parameters for seed culture S1 (pH 7.36, PMV% 10) and S2 pH 7.16, PMV% 14) was shown in Table 5. On 36 h maturation, seed culture was transferred into production flask.

3.3.3. Analysis of ascomycin production in shake flasks samples through HPLC

Concentration of ascomycin in the fermentation broth was calculated by comparison of peak areas of sample with that of ascomycin standard. The typical chromatogram of ascomycin pure and fermentation broth from shake flask experiment are presented in Fig. 4A and B respectively.

In present study shake flask was performed using eight different selected production media. As discussed earlier, the experiments were performed in two ways.

In two stage fermentation, significantly higher bioactive metabolite was obtained in P3 medium (0.216 mg/g) followed by P8 medium (0.172 mg/g) on 11th day of incubation, whereas in remaining media, very less ascomycin concentration was observed that range from 0.001 to 0.004 mg/g (Table 4).

In three stage fermentation, secondary metabolite production was largely affected by adding one more seed stage. Fig. 5 showed that different production media had varied effect on metabolite production. The increment in the yield was remarked after employing two seed stage in shake flask process (Fig. 5). The impact, on yield, of S2 seed culture was more effective in comparison with S1 seed culture. Out of eight, it was found that P3 (0.296 mg/g) and P8 (0.356 mg/g) media were supported the maximum bioactive metabolite production with S2 seed culture. On other hand, with S1 seed culture, P3 (0.199 mg/g) and P8 (0.201 mg/g) media showed moderate amount of ascomycin production. Less than 0.1 mg/g productivity was noted in remaining media (P1, P2, P4, P5, P6 and P7) with both seed culture S1 and S2. To improve the ascomycin titer, further experiments were designed with S2 media to investigate the optimum age of seed culture. On the basis of obtained yield two production media P3 and P8 were screened for further study.

3.3.4. Determination of incubation age of seed culture

The experiment was further simplified to investigate the impact of the incubation age of seed culture on the fermentation. For the production of secondary metabolites, it is necessary to determine the optimum age of seed culture. Seed culture at the age of 36 h, 40 h, 44 h and 48 h was transferred in production media. Fig. 6 depicted the growth profile of seed culture.

The impact of seed culture age in both media (P3 and P8) for ascomycin production was summarized in Fig. 7. Maximum concentration of ascomycin (0.5 mg/g) was recorded in P8 media followed by P3 (0.365 mg/g) on 11th day of cultivation when 44 h matured seed culture (S2) was transferred and then production was declined by using 48 h grown seed culture. 36 h and 40 h grown seed culture showed comparatively less production of bioactive metabolite. P8 media supported the maximum production of ascomycin with S2 seed. Amendment of 44 h grown S2 seed culture enhanced the secondary metabolite production while 36 h, 40 h and 44 h seed culture showed moderate activity in P8 media.

4. Discussion

Interactions between microorganisms and growth media have long been known to play an important role in determining the rate of production and yield of metabolites. Microorganisms grow on or in growth medium which supply nutrients required by the organism. Enrichment of culture is important for proper growth of microorganisms. It is necessary to provide carbon sources, nitrogen sources, minerals, 'salts for osmotic balance of culture' and water for enrichment of microbial culture. The production of specific metabolites in high titer could be possible by maintaining proper control and regulation at different levels *via* transport and metabolism of extra-cellular nutrients, precursor formation and accumulation of intermediates (Rokem et al., 2007). Two development phases are normally recognized in microbial culture for producing secondary metabolites *i.e.* a vegetative phase (trophophase) with vigorous growth and negligible metabolite production and fermentation phase (idiophase) where culture is stationary and metabolite production initiated (Giancarlo and Rolando, 1993).

In the study presented here, P3 medium was taken from literature (Petkovic et al., 2011). In P3, the carbon sources were dextrose, dextrin white and glycerol, while soya-flour and soya-peptone were nitrogen sources supplemented with lysine. Lysine being an important precursor in ascomycin pathway, its supplementation had positive effect on the titer of ascomycin. Soya flour, a rich in lysine, hence was recommended in the media. In P8 medium, designed in-house, the carbon sources were corn starch, malto-dextrin, glycerol and maltose while nitrogen sources were soya-flour and dry yeast. The soya flour, a rich source of lysine and dry yeast had high content of amino acids helped cell growth

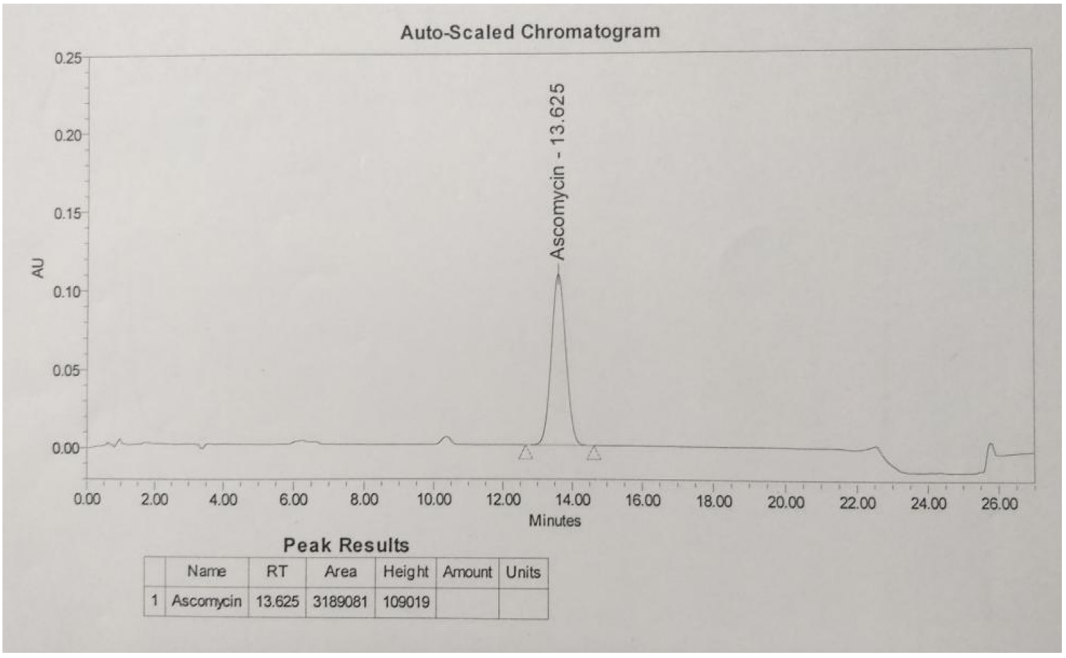

Fig. 4A. Chromatogram of ascomycin standard.

thus increasing the titer of ascomycin in P8 medium.

In a fermentation process, composition of culture medium plays a major role and will determine the level of end product. Nutrients required for fermentation media depend upon the organisms as well as the type of fermentation process to be used. Poor choice of fermentation media might result in poor yield or output. So, types of nutrients present in the fermentation media always determine the yield of the product.

Filamentous organisms when grown in submerged culture, exhibit different morphological forms ranging from dispersed individual mycelial elements to densely interwoven mycelial masses referred to as pellets (Papagianni, 2004; Nielsen, 1996). Under conditions of rapid growth and high branching frequency, filamentous organisms may produce pellets (Bader, 1986).

Table 4
Screening of different production media.

Sr. No.	Production media	Activity (mg/g)
1	P1	0.001
2	P2	0.004
3	P3	0.216
4	P4	0.002
5	P5	0.004
6	P6	0.002
7	P7	0.003
8	P8	0.172

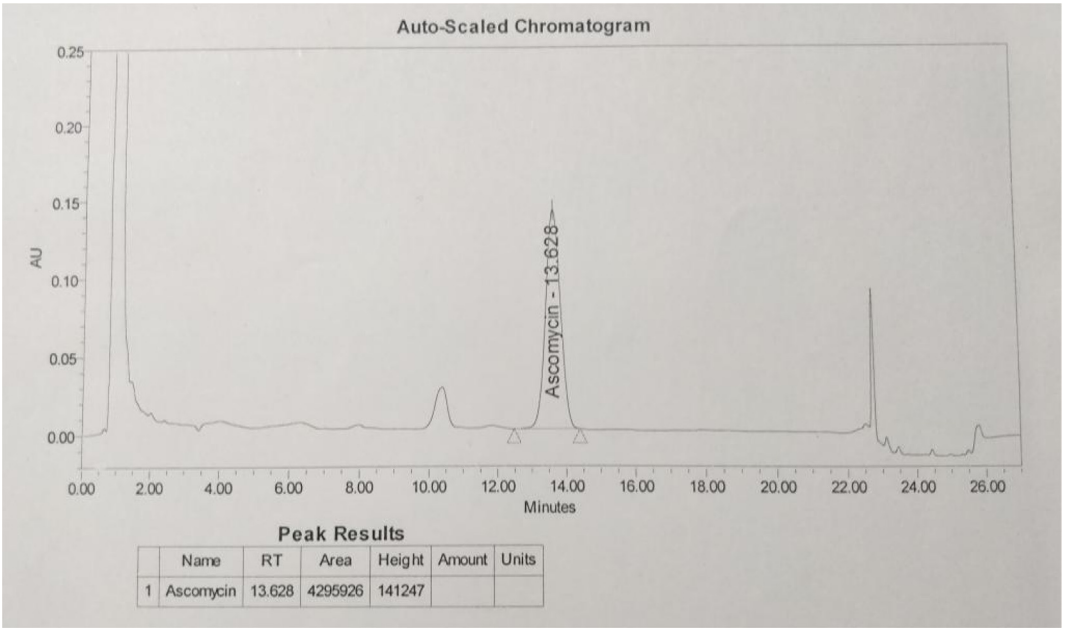

Fig. 4B. Chromatogram of shake flask broth.

Table 5
Transferring details of seed culture.

Parameters	Seed culture	
	S 1	S 2
Age (hr)	36	36
pH	7.36	7.16
% PMV	10	14

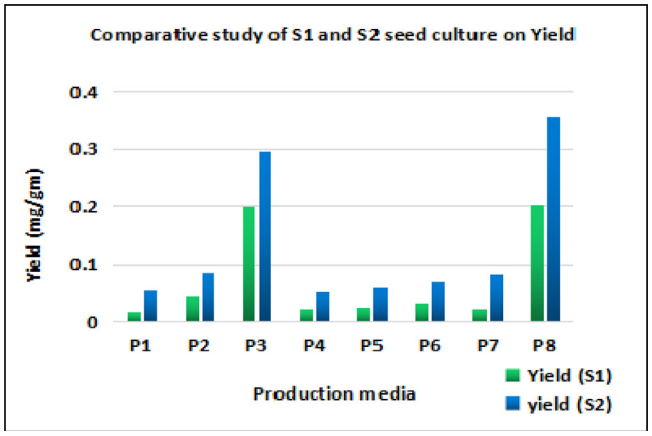

Fig. 5. Impact on yield after addition of seed stage.

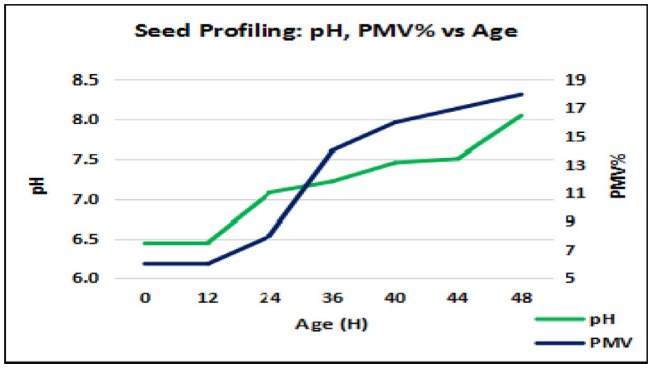

Fig. 6. Seed profiling.

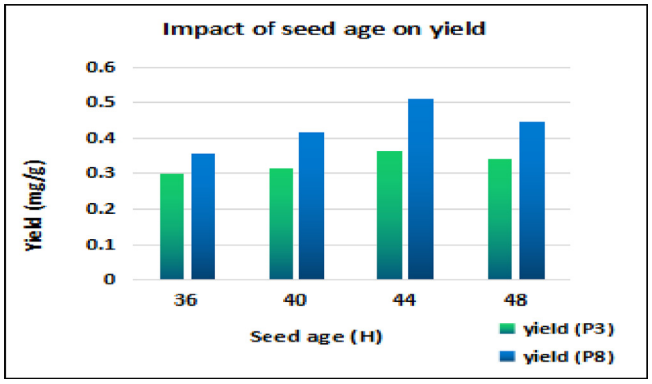

Fig. 7. Yield of Ascomycin in P3 and P8 medium at different age of seed culture.

Streptomycetes possess a huge potential to produce valuable natural products. In this study *Streptomyces ascomyceticus* was evaluated for the production of ascomycin. *Streptomyces ascomyceticus* is a gram positive, aerobic, filamentous bacteria which produces well developed vegetative hyphae with branches and forms interwoven network of mycelia. The mycelial growth pattern produced in P8 medium was in clumped form, consisted of entangled hyphae, while in P3 medium, pelletal manner of mycelial growth was observed. The voulme of mycelia gradually increased with age resulting in dense network of hyphae. Cell density indicated how the culture would behave in terms of growth and product formation. At early age, the branches of hyphae observed were thick and smooth which became thin and dense which at the late stationary phase attained club shaped structure.

In conventionl approach, usually concentrate initially on optimizing the final fermentation stage but understanding the seed stage can also improve the final stage productivity (Warr et al., 1996). The study presented herein, two methods were employed for ascomycin production in shake flasks. The first method contained two stage where grown lab inoculum was transferred into production medium and in the second method, one more stage was added where grown lab inoculum was transferred into seed media then to the production media. Seed medium was required for the increment of inoculum size and to minimize the lethal effect of cells. The physiological state of seed culture including metabolic activity and morphological structure were the important factors for the efficient production of bioactive metabolites (Zou et al., 2011).

In the first set of experiments, the seed culture contributed to long lag phage in fermentation resulting in late logarithmic phase and hence took longer time to produce ascomycin resulting in low titer. While in the second set of experiments it was observed that lag phase was shorter in production as inoculum was habituated in seed media and thus desired cell density was obtained leading to shorter logarithmic phase in production and thus more titer. The similar opinion was put forth by Rajnisz et al. (2015) in their study in which they mentioned that the 72 h seed culture needed more time to adapt to new conditions resulting delay in reaching the maximum activity of secondary metabolites. In addition a low inoculum density may give insufficient biomass causing reduced product formation, whereas a higher inoculum concentration may produce too much biomass and deplete the substrate of nutrient necessary for product formation (Ellaiah et al., 2004).

5. Conclusion

The study on growth of the culture and screening of fermentation media is a crucial step for bioactive metabolite production. In this study two stage and three stage fermentation process have been discussed and reviewed. Three stage approach was found to have more impact to maximize the titre of ascomycin. Physcial parameters (pH, PMV% and microscopy) were observed at different intervals to determine its impact on productivity. On the basis of results obtained, P3 and P8 media were selected to perform further experiments. 44 hrs grown S2 seed gave higher yield on 11th day of cultivation in P8 production media. Maximum 0.5 mg/g of ascomycin was obtained in second set of experiments, whereas 0.2 mg/g ascomycin concentration was achieved in set 1 experiment. Thus it can be concluded that the “three stage fermentation” was better over the “two stage fermentation” process. Further experiments need to be planned for medium optimization and culture selection to achieve the higher titer of ascomycin than obtained by this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101159.

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