



Analysis and evaluation of penicillin production by using soil fungi

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ABSTRACT

Penicillin is an antibiotic drug effective against many serious diseases such as syphilis and Staphylococcus infections. Different kinds of fungi have used to obtain various biotechnological products. The current study elucidates the qualitative and quantitative analysis of Penicillin derived from soil fungi using rapid High-performance liquid chromatography (HPLC) when precreating on different growth conditions. The samples are collected and render with HPLC after penicillin produced in both synthetic and semi-synthetic media, over a regular period of incubation. A significant effect on penicillin production has seen in nutrient media or growth media. Our results strongly indicate that the synthetic medium, used the nutrient source as a pure chemical source is good and consistent in penicillin production from 4 to 5 days of incubation. However, systematic, undefined, and rather cheap potato dextrose agar medium is low in penicillin productivity. Penicillin antimicrobial activity was tested on the normal flora of human intestine by taking *E. coli*, *Enterococcus*, and *Bacillus* as indicator organisms and the research results suggest that Penicillin is a potent antibiotic even against the helpful normal flora of the human intestine.

1. Introduction

Fungi are a high-priority part of soil microbiota. The fungi have a prime role to caper in the soil ecosystem and they execute ecological amenity that strongly impacts the quality of human life and have immense potential for providing economic repose, e.g., the isolation and identification of the soil fungus Penicillium leading to a large pharmaceutical industry of antibiotics (Ainsworth and Bisby, 1995 - Diana, 1994). Fungi can produce dissimilar secondary metabolites with antibacterial activity against the diversification of microorganisms. Of these metabolites, penicillin is a starting point for the detection of highly effective antibiotics, a principal step in therapeutic medicine (Peñalva et al., 1998 - Brakhage et al., 2004). There are about 1.5 million species of fungi (Dayalan et al., 2011). They are involved in various ecological services that have eventual to provide different economic interest, e.g., e.g., the isolation and identification of the soil fungus Penicillium

leading to a large pharmaceutical industry of antibiotics (Hawks worth and Rossman, 1997). The demand for penicillin is increasing at exorbitant rapidity. Penicillin belongs to a group of antibiotics which is derived from Penicillium fungi (Diana, 1994). Penicillin count several members with different antimicrobial spectrum or activity (Samanidou et al., 2007). The Penicillin antibiotics have been crucial since ancient times as they were the first drugs that were effective against many serious diseases like syphilis and Staphylococcus infections (Andersen and Frisvad, 1994). Even today Penicillin antibiotics are in demand (Quinn, 2013; Ferech et al., 2006). All penicillin is Beta-lactam antibiotics and is used in the treatment of bacterial infections caused by susceptible, usually Gram-positive, organisms (Kong et al., 2010). In this study, qualitative and quantitative analysis of Penicillin, derived from the soil fungi using Reverse phase HPLC (High-Performance Liquid chromatography) was done. Different nutrient media (synthetic and semi-synthetic) were selected to grow the isolated soil fungi and to

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Table 1
Samples selected for the analysis of Penicillin production.

No.	Samples	Description
1	PDB-I	Sample extracted from the PDB after 3 days of incubation
2	SB-I	Sample extracted from the synthetic broth after 3 days of incubation
3	PDB-II	Sample extracted from the PDB after 4 days of incubation
4	SB-II	Sample extracted from the synthetic broth after 4 days of incubation
5	PDB-III	Sample extracted from the PDB after 5 days of incubation
6	SB-III	Sample extracted from the synthetic broth after 5 days of incubation

Note: PDB: Potato Dextrose Broth, SB.

produce Penicillin into the liquid medium. One in typical with semi-synthetic, fungal growth media and the other in industrial production with defined media. Furthermore, the evaluation of penicillin production in their respective media was investigated. *Penicillium chrysogenum* is a commonly occurring mold in indoor environments such as dust, indoor air, and damp building materials (Chang et al., 1995; Gravesen, 1999), (Hunter and Lea, 1995). Furthermore, *P. chrysogenum* is frequently identified as a food spoilage agent and has gained much attention for its use in the production of the antibiotic penicillin (Samson et al., 1977). The taxonomy of this species was studied extensively by Raper & Thom (1949) and they accepted four species in the “*Penicillium chrysogenum* series”: *P. chrysogenum*, *P. notatum*, *P. mele grinum*, and *P. cyaneofulvum*. However, Samson et al. (1977) regarded this series as one broad species and did not accept Raper and Thorn’s “*P. chrysogenum* series”. More recently, other species have been included in *Penicillium* series *Chrysogena*, such as *P. flavigenum*, *P. nalgiovense*, and *P. dipodomyis* (Frisvad et al., 1987; Banke et al., 1997). Taxonomy of *Penicillium chrysogenum* and related xerophilic species, based on isozyme analysis (Frisvad and Samson, 2004), and these species are different from *P. chrysogenum* although all species produce penicillin (Frisvad et al., 1987). Penicillin production can be highly useful for the food industry and widely recommended to have production high.

2. Material and methods

2.1. Chemicals

Soil, potato dextrose agar media, pipettes, petriplates, 9 ml dilution blanks, inoculating loop, sterile petriplate, potato dextrose agar.

2.2. Isolation of microorganisms

The soil samples were processed using the soil dilution plate in potato dextrose agar according to the method of (Waksman, 1922).

2.3. Preparation of pure culture penicillin by pour plate method

A standard volume of sample was first plated on which melted and cooled nutrient agar was added and the plates are rotated clockwise and anti-clockwise. Each organism in the sample was separated from all others. When the agar solidifies, the cells were trapped in the agar and developed into colonies. In brief, an aliquot of the diluted sample was placed in an empty sterile plate and poured in 15 ml of melted agar which has been cooled to 15 °C, and was swirled to mix well. It was cooled undisturbed to solidify on a flat table top. Then, it was incubated to develop colonies at 30 °C.

2.4. Penicillin production

Penicillin produced in two different liquid media i.e. regular fungal growth medium and a completely defined synthetic medium is

Table 2
Composition of synthetic penicillin production medium (100 ml) preparation.

No.	Chemical Ingredients	Quality	No.	Chemical Ingredients	Quality
1	Lactose	3.0 g	8	Ammonium phosphate	0.1 g
2	Glucose	1.0 g	9	Copper sulphate	0.001 g
3	Citric acid	1.0 g	10	Iron sulphate	0.002 g
4	Acetic acid	0.25 g	11	Zinc sulphate	0.001 g
5	Phenyl acetic acid	0.05 g	12	Manganese sulphate	0.001 g
6	Ammonium phosphate	0.5 g	13	Distilled water	100 ml
7	Magnesium sulphate	0.05 g			

Table 3
Composition of potato dextrose broth.

No.	Chemical Ingredients	Quantity
1	Potato extract	20.0 g
2	Dextrose	2.0 g
3	Distilled water	100 ml

considered as two different treatments for the analysis of their respective Penicillin production abilities derived from HPLC analysis. The sample or treatments selected for the research program are given in Table 1.

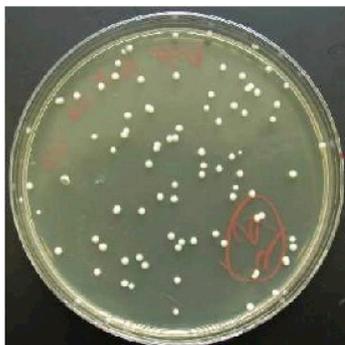
The graphic representation for the above samples via HPLC is in Graph Nos. 1, 2, 3, 4, 5, 6.

For the preparation of the synthetic medium for Penicillin production, the media where all the ingredients are pure chemicals, so the ingredients form a right mixture of requirement, and it is believed to be supporting the microbial growth at the fullest extent. It is also called synthetic media, and the ingredients are as listed below in Table 2.

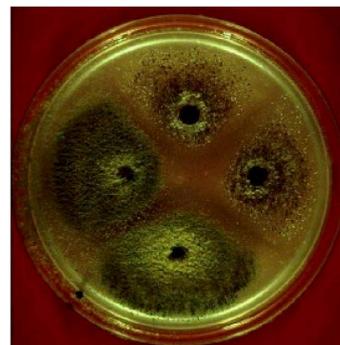
For the preparation of the semi-synthetic medium for Penicillin production, the regular fungal medium used is PDB (liquid) media, and it was prepared by using the potato starch extract added with other sources of sugar for fungal growth. We can get the same by using ready use medium from HIMEDIA manufactures and can be prepared by mixing it suitable quantity of water. The composition of PDB is listed in Table 3.

2.4.1. Isolation and identification of fungal isolates

The soil fungi were isolated by both the Direct Soil Inoculation and the Soil Dilution Techniques using the pour plate method. The Media used for the isolation were Potato Dextrose Agar (PDA). Pure cultures of fungal isolates were identified using both macroscopic (cultural) and microscopic (morphological) features concerning (Barnett and Hunter, 1998) and (Domsch et al., 1980). Several antibiotic drugs have been discovered from soil-inhabiting microorganisms which include fungi (20% of isolated antibiotics), actinomycetes (70%) and eubacteria (10%) (Bredy, 1974) and (Lechevalier, 1975). The last three decades are characterized by the novel discoveries of microorganisms capable of producing compounds, as a potential source of new antibiotics (Antibi and Fishlock, 1986). Pure culture has been taken with the help of inoculating needle teasing some portion of the growth of the fungus and places it on the slide. If the fungus is heavy sporing like *Aspergillus* or *Penicillium* washing with 70% ethanol., removing ethanol by blotting paper. Then by dropping a drop of Lacto-phenol cotton blue, spreading the mycelium with needles, place a coverslip. Examine, note morphology of hyphae, spore structures, etc. Yeast, a type of fungi (plural for fungus), is found in many places from nature, to research labs and even everyday kitchens for baking. Yeast colonies generally look similar to bacterial colonies. Some species, such as *Candida*, can grow as white patches with a glossy surface.



Round yeast colonies



Aspergillus



Pink yeast colonies

2.4.1.1. *Molds*. Molds are fungi, and they often appear whitish-grey, with fuzzy edges. They usually turn into a different color, from the center outwards. Two examples of molds were shown below:



Black Mold

2.4.1.2. *Other fungi*. Moss green colonies, a white cloud, or a ring of spores can be attributed to the growth of *Aspergillus*, which is common in such fungal infections as athlete's foot. Here is an example of what *Aspergillus* looks like:

2.5. Qualitative and quantitative analysis by HPLC

HPLC method was employed for analysis with the C18 stationary phase column and mobile phase with Acetonitrile and Phosphate buffer added with Hexane sulphonic acid. The utility of HPLC as an analytical tool was performed for the qualitative and quantitative analysis of antibiotic Penicillin produced where the different source of nutrition is supplied in the growth medium. The chromatographic system selected for the study is as follows:

2.6. Testing of antibiotic activity on intestinal flora

The liquid cultures of intestinal indicator bacteria of non-pathogenic strains i.e. *E. Coli*, *Enterococcus faecalis* and *Bacillus subtilis* were incubated with a standard concentration of Penicillin (0.1 mg in 1 ml of aqueous suspension mixed in the nutrient broth flasks). There were three treatments of Penicillin with three different cultures to understand the efficiency and effect of Penicillin over each of them. The liquid culture flasks added with standard quantity (1 ml each infusion of each culture) into respectively labeled culture flasks. The liquid broth cultures are kept in a shaking incubator controlled at a mesophilic temperature 35 °C. The culture flasks were removed from the incubator after 24 h, 48 h, 72 h, and 96 h of regular intervals of their growth for observation. The respective treatments from different flasks were taken (10 ml each, 3 ml of cuvette capacity) and absorbance were recorded at 600 nm in a UV/Vis Spectrophotometer. The absorbance represents the turbidity developed by the growth of the cultures incubated and more the absorbance observed is the sign of much the growth against the antibiotic effect of Penicillin treatment applied. Three of the solid culture treatments in liquid suspension prepared by mixing one loop-full of pure culture with 10 ml sterile distilled water and were developed on nutrient agar plates by spread plate technique using L shaped glass rod in aseptic conditions inside a laminar flow bench. Each of the plates is bored with a sterilized borer which can hold a standard volume of the pure antibiotic (Penicillin) suspension in tenfold serial dilution. There were nine bores provided in three agar plates allotted for every single treatment of three of the cultures to establish the antibiotic activity over each of the treatments. The standard volume of the antibiotic (0.1 ml) was dispensed into each of the labeled bores with specified dilution from 10⁻¹ to 10⁻⁹ concentrations into respective treatments having the spread cultures of *E. Coli*, *Enterococcus*, and *Bacillus* species. These inoculated plates were allowed to absorb the antibiotic suspension for 15 min and were kept in an incubator at 300C. Then the plates were observed in one-day intervals for the antibiotic zones. If the antibiotic was effective against the antibiotic that was expressed by a clear zone formed around the bore loaded with the antibiotic. All the antibiotic zones formed were measured by the unit scale in centimeters (cm).

Table 4

No.	Components	Identity
1	Cartridge	250 × 4.6 mm-Bondapak C18 (a reverse phase column)
2	Pump	Waters 515, Isocratic system
3	Detector	Waters-W 2487, UV/Visible
4	Injector	Rheodine, Manual
5	Column	ACE, C18 Reverse phase, silica bonded
6	Pump Control Module	II
7	Filters	
	Mobile phase filtration	I paper, stainless steel pump inlet filters
	filtration	2 µm cellulose nitrate syringe disc filters
		Pre-column inline secondary filter
8	Software	Empower-II

Table 5

Quantitative data of critical parameters obtained from the chromatograms (Presented in Appendices).

S. No.	Name of the Sample	*RT (min)	#PA (µV X sec)	PH (µV)	~PW (sec)
1	Std 1	1.692	64620850	3201720	41
2	Std 2	1.679	58782882	3103102	37
3	Std 3	1.692	65056111	3214900	40
4	PDB- I-1	1.595	1314419	65414	39
5	PDB- I-2	1.595	1340183	66009	32
6	PDB- I-3	1.622	710376	53664	39
7	SB-I-1	1.571	575435	42239	42
8	SB- I-2	1.626	845541	61426	39
9	SB- I-3	1.539	154031	13284	39
10	PDB- II- 1	1.545	267577	23005	40
11	PDB- II- 2	1.545	267577	23005	41
12	PDB- II- 3	1.546	239773	23200	32
13	SB- II- 1	1.553	332539	27224	39
14	SB- II- 2	1.545	240162	22586	39
15	SB- II- 3	3.742	8339767	228382	41
16	PDB- III- 1	1.618	539299	34495	41
17	PDB- III- 2	2.281	193128	12296	42
18	PDB- III- 3	2.276	175419	11115	39
19	SB- III- 1	3.736	7472018	218514	40
20	SB- III- 2	3.728	7051660	214099	41
21	SB- III- 3	3.742	8339767	228382	40

Note: *RT- Retention Time.

#PA- Peak Area.

PH- Peak Height.

~PW- Peak Width.

The statistical analysis data for the mean, standard deviation (SD) and relative standard deviation (RSD) values acquired from the data are provided in Table 5.

3. Results and discussion

Several high-production strains have been described that comprise multiple copies of the penicillin cluster (van den Berg, 2010). Protection anticipation presumption was compulsory to avoid interference of the macromolecules adjacent in soil fungi with active-matrix sites (Quesada-Molina et al., 2012). In general, the deduction of penicillin from soil fungi is included in multi residual and multiclass methods, which include different stages of purification, such as defecation, centrifugation, dilution and less selective SPE protocols (Evaggelopoulos and Samanidou, 2013; Berendsen et al., 2013; Becker et al., 2004; Junza et al., 2014; Rezende et al., 2012). Thus, the present experiments were considered successful, as the goal of the study was to develop a secure, durable, reasonable, and simple method for the analysis of penicillin from soil fungi. The obtained recoveries were similar to or lower than those obtained by Ghidini et al., who used only sample (acidic) precipitation, centrifugation, and filtration (Ghidini et al., 2003).

3.1. Penicillin extraction

A four-step solvent transfer method was used for the extraction of

Table 6

Statistical analysis of data from the chromatograms.

S. No	Name of the sample	Mean	*SD	#RSD
For RT				
1	Std	1.687667	0.007506	0.44473
2	PDB-I	1.604	0.015588	0.971849
3	SB-I	1.578667	0.044004	2.787402
4	PDB-II	1.545333	0.000577	0.037361
5	SB-II	2.28	1.266135	55.53226
6	PDB-III	2.058333	0.381348	18.52703
7	SB-III	3.735333	0.007024	0.188036
For PA				
1	Std	62819948	3502968	5.576204
2	PDB-I	1121659	356414.7	31.77566
3	SB-I	525002.3	348502.7	66.38117
4	PDB-II	258309	16052.65	6.214513
5	SB-II	2970823	4649872	156.518
6	PDB-III	302615.3	205165.2	67.79737
7	SB-III	7621148	656875	8.619108
For PH				
1	Std	3173241	61098.3	1.925423
2	PDB-I	61695.67	6961.987	11.2844
3	SB-I	38983	24235.6	62.16966
4	PDB-II	23070	112.5833	0.488007
5	SB-II	92730.67	117500.4	126.7115
6	PDB-III	677944	13170.77	1.942752
7	SB-III	220331.7	7312.931	3.319056
For PW				
1	Std	39.33333	2.081666	5.292371
2	PDB-I	36.66667	4.041452	11.02214
3	SB-I	40	4.358899	10.89725
4	PDB-II	37.66667	1.732051	4.598365
5	SB-II	37.66667	5.131601	13.62372
6	PDB-III	39.33333	0.57735	1.46784
7	SB-III	37.33333	1.732051	4.639422

penicillin from different shake flask cultivation systems. Briefly, first penicillin produced in shake flask culture was extracted; into amyl acetate (D. Rowley et al., 1946) is added and then transferred from amyl acetate into phosphate buffer. In the third step, extraction was made from a buffer solution into chloroform and finally transferred from chloroform into the water. The extracted material was soaked into sterilized filter paper discs for quantitative analysis (see Table 4).

3.2. HPLC analysis of purified penicillin

The HPLC is used as an analytical tool for the quantitative analysis of antibiotic penicillin produced where different media are used (Jehl et al., 1985). HPLC analysis shows that there is a significant effect of nutrient media or growth media composition in the production of penicillin. The results obtained from the data generated in the respective chromatogram by HPLC strongly indicate that the synthetic medium where the pure chemical is used as a nutrient source is good and consistent in penicillin production from 4 to 5 days of incubation. In contrast, the disciplined, undefined, and rather a cheap potato dextrose agar medium is low in penicillin productivity. The quantitative data obtained is provided in Table 5.

The assay values of each of the four different treatments of growth medium (PDB and SB at 3, 4 and 5 days of incubation) are calculated according to the formula and their respective assay values are recorded. The assay values are calculated based on the peak areas integrated with chromatograms, sample weights and a standard assay of RS. Comparisons made considering the assay values of RS with treatments. The concentration values are recorded thereof with the different samples in the respective culture media. The comparative figures are presented in Table 6.

A line diagram to offer the graphical representation depicting the above facts and figures has been drawn by which one can easily understand the efficiency of production of Penicillium fungus in releasing Penicillin into two different kinds of medium, one is laboratory culture

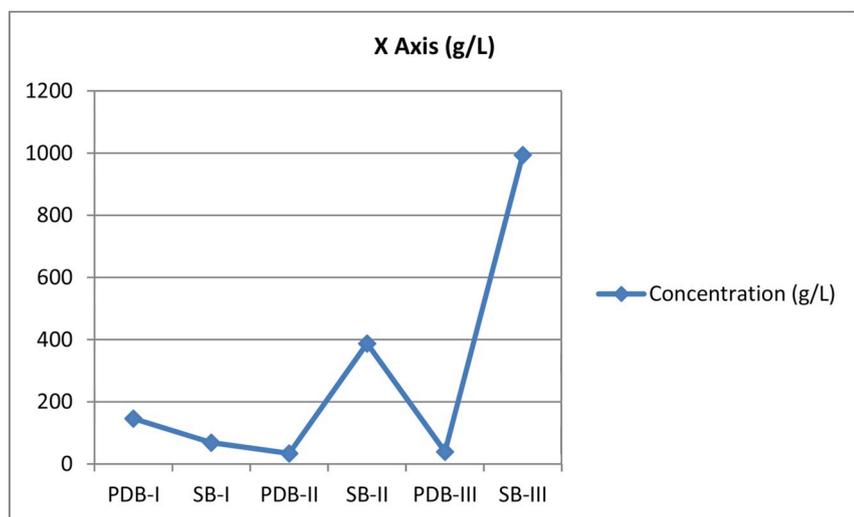


Fig. 1. Comparison between the different pH treatments in terms of ass.

Note: On X-axis- treatments.
On Y-axis- Assay values (mg/100 mg of sample).

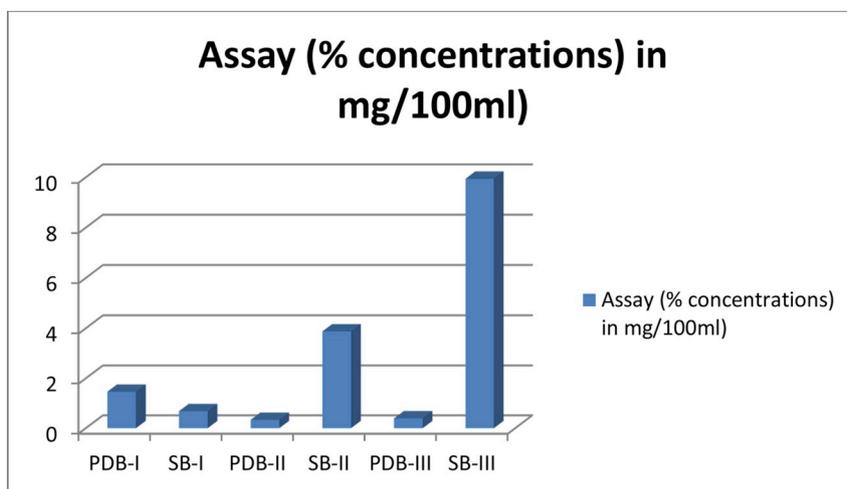


Fig. 2. Assay comparison between pH treatments of Penicillin.

Table 7
Concentration comparison between pH treatments of Penicillin.

S. No.	Name of the sample	Assay (% concentrations) in mg/ 100 ml)	Concentration (g/ L)
1	PDB-I	1.46340776	146.3408
2	SB-I	0.68496063	68.49606
3	PDB-II	0.33701088	33.70109
4	SB-II	3.87597626	387.5976
5	PDB-III	0.39481651	39.48165
6	SB-III	9.94316838	994.3168

medium and the other is synthetic production medium. The same is presented in the graph (Fig. 1) below.

The same can also be understood by plotting a bar diagram for the concentrations from the samples collected from the culture media at regular intervals (Fig. 2). The mean Retention time values generated by

Table 8
Absorbance of cultures (treatments) in liquid NB media.

S-NO	Name of the organism	Absorbance (AU) - 1st day	Absorbance (AU) - 2nd day	Absorbance (AU) - 3rd day	Absorbance (AU) - 4th day
1	<i>E. coli</i>	0.06	0.154	0.273	0.206
2	<i>Enterococcus</i>	0.052	0.154	0.346	0.082
3	<i>B. subtilis</i>	0.014	0.522	0.695	0.671

different peaks in the chromatograms are compared to the deviation among them by calculating their Standard Deviations (SD). The values are presented in Table 6.

3.3. Antimicrobial activity testing of penicillin antibiotic on normal flora of human intestine

3.3.1. Activity testing in liquid cultures

The absorbance represents the turbidity developed by the growth of the microbial cultures incubated in the liquid broth (see Table 7). More the absorbance observed, is a sign of much the growth of the culture and less the activity of antibiotic inhibition. The observation in terms of absorbance for treatments (three culture flasks) of Penicillin is presented in Table 8.

The dynamics of these results in absorbance can be easily understand by plotting the results in a line diagram (graph) keeping four

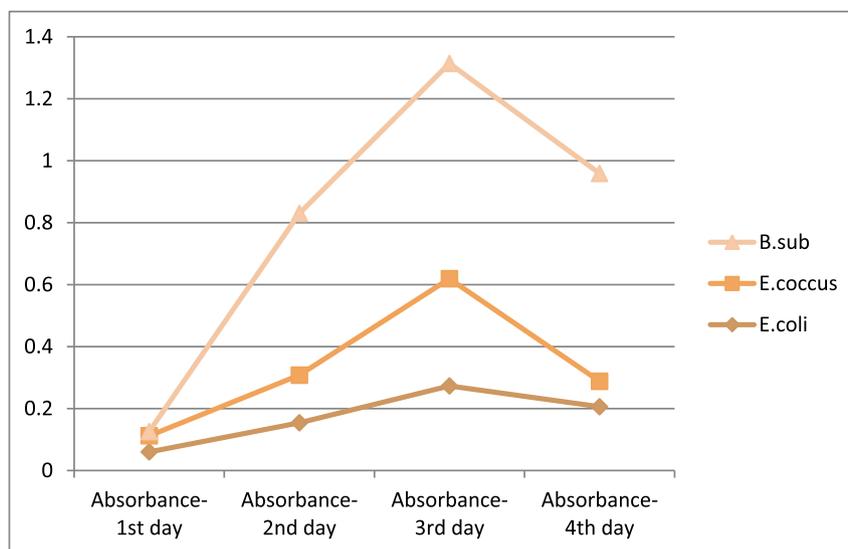


Fig. 3. Graph: Growth patterns of three treatments according to the Absorbance at 600 nm added with Penicillin.

Table 9

Antibiotic activity by the zone reading.

S. No	Name of culture	Maximum dilution showing clear zones	Antibiotic activity
1	<i>E. coli</i>	10^{-8}	11.11111
2	<i>Enterococcus</i>	10^{-9}	14.28571
3	<i>B. subtilis</i>	10^{-7}	12.5

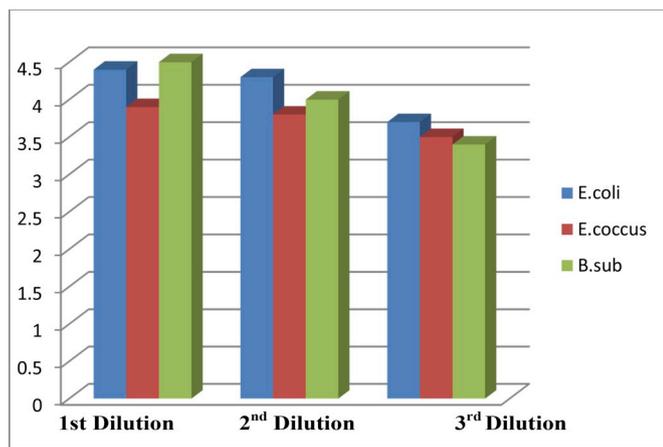


Fig. 4. Antibiotic activity pattern of Penicillin in solid media treatments.

observations (four days) on X-axis and absorbance at 600 nm on Y-axis for the three sets of treatments (microbial cultures) with three different intestinal flora *E. coli*, *Enterococcus* and *Bacillus* (Fig. 3).

3.3.2. Activity testing on solid cultures (agar cultures)

As discussed in the method part, the agar cultures of the treatment are observed for the zone formation at provided different tenfold dilution of the antibiotic. The maximum dilution which has shown the clear zone formation is the sign of the effectiveness of the antibiotic over that particular culture. Hence the antibiotic activity of Penicillin upon culture can calculate by using the standard formula.

Antibiotic activity = Reciprocal of maximum dilution showing clear zones X100/Volume of antibiotic applied (ml)

By the above expression of formula, the antibiotic activities in different antibiotic dilutions are calculated and are provided in Table 9.

The bacterial inhibition of Penicillin is directly proportional to the diameter of the antibiotic zone formed, and their antibiotic activity is calculated based on the formula stated above. The dynamics of their respective antibiotic response with Penicillin is plotted in the bar diagram.

Validation of Samples and Peak Summary by Analytical Performance of HPLC.

The combination of this fast extraction approach with HPLC determination resulted in an affordable analytical method with good performance in terms of linearity and precision.

3.4. HPCL chromatograms graphs

(Fig. 4).

4. Conclusion

Currently, simple and rapid methods have been developed to screening microorganisms for high yield antibiotic ability to require of new antibiotics. Moreover, the antibiotic-producing organism has been isolated from soil samples. In our current study, we show Penicillin producing fungi (*Penicillium*) was grown into pure cultures from the soil, identified and transferred to two different test conditions. HPLC analysis was done after the collection and extraction of samples from these culture media at regular intervals from three to five days. Our results clearly show that there is a significant effect of using synthetic medium over the conventional laboratory semi-synthetic growth medium in the production of Penicillin. Therefore, it can be recommended for the mass production of penicillin even in the laboratory scale but not the Potato dextrose medium Penicillin antimicrobial activity testing on normal flora of human intestine by taking *E. coli*, *Enterococcus* and *Bacillus* as indicator organisms suggests that Penicillin is a potent antibiotic and its activity is observed at best in the normal intestinal flora. Furthermore, our observation and results in both liquid and solid cultures depict that they follow the regular growth curve pattern while inhibited by the antibiotic. The activity of Penicillin, however observed maximum with *Bacillus* cultures followed by *E. coli* and then *Enterococcus*. So the usage of Penicillin as a regular antibiotic dose can be restricted as it possesses a significant detrimental effect on the growth of the normal intestinal flora which is crucial and useful for the regular digestion process and conditioning of human intestine.

Conflicts of interest

The authors also declare that there is no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bcab.2019.101330>.

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