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Original Article

Exploration of potent soil bacteria for Menaquinone-7 production

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Abstract

The present study aimed to isolate and screen the bacteria from soil origin for the production of Menaquinone-7 (MK-7). In this approach, a total of 140 bacteria were isolated from the soil samples collected from different fields of Gulbarga region, Karnataka, India. Further, all the bacterial isolates were investigated for MK-7 production under submerged fermentation. A total of 60 isolates were found to produce MK-7. The productivity of MK-7 by these isolates were qualitatively analyzed and identified by TLC. The quantitative estimation of MK-7 by these isolates revealed that, the isolates viz. KLSG-9, KLSG-10, KLSG-5, KLSG-1 and KLSG-11 were noticed with the good yield. Amongst these five isolates, comparatively the isolate KLSG-9 was found to be the highest MK-7 producer with the yield of 48 mg/l.

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Key words: MK-7, Menaquinone 7, Vitamin K2, Osteoporosis.

1. Introduction

Vitamin K is a fat-soluble compound and was identified as anti-hemorrhagic factor more than 85 years ago. It is capable in correcting dietarily induced bleeding disorders in chickens [1]. It is also an essential cofactor required for the post translational conversion of glutamic acid residues of specific proteins in the blood and bone into gamma-carboxyglutamic acid (Gla) [2]. In nature, this vitamin occurs in two forms, phylloquinone (vitamin K1), which is present in green plants; and menaquinone (MK) (vitamin K2), which is produced by intestinal bacteria [3,4,5]. The menaquinones have variable side chain lengths of 4-13 isoprene units by which they derived their name as MK-1 (which bears 1 isoprene unit), MK-2 (bearing 2 isoprene units) like this upto MK-13 [6]. Among menaquinones, MK-7 is a highly bioactive homologue of vit-K [7] and it is abundantly present in fermented soybean food products (natto) [8].

MK-7 gained unique place in pharmaceuticals as it has significant effect on preventing osteoporosis, cardiovascular diseases & blood coagulation [9, 10, 11]. Consumption of MK-7 reduces coronary/artery calcification and could protect against cardiovascular diseases [9]. In recent years there is increase in number of patients with osteoporosis & cardiovascular diseases. On the other hand researchers being involved in determining the alternative approaches to prevent these health complications [12]. According to Tsukamoto, the intake of MK-7 stimulates carboxylation of osteocalcin which plays a critical role in bone formation [6]. The likely therapeutic dose of MK-7 for treatment of osteoporosis and cardiovascular diseases is from MK-7 rich dietary sources (i.e., blue cheese, meat and fermented soybean) would require the consumption of impractically large quantities [12]. It is therefore, desirable to develop a rich source of MK-7 as a dietary supplement.

MK-7 is produced mainly by liquid state fermentation using Bacillus sp. such as Bacillus subtilis and Bacillus amyloliquefaciens BY01 [13,14] and also can be obtained from food products such as cheese, meat and fermented soybean food product in Japan; it contains comparatively high amounts of MK-7 (About 800-900 microgram/100g natto) compared with other foods [15]. Apart from these, other bacteria such as lactic acid bacteria are reported to produce MK-7 [16].

The current challenge for MK-7 fermentation involves increase in the yield of fermentation to make large scale production viable and to screen new species [17]. Therefore, to meet the global requirement, efforts are required to isolate a potent strain for production of MK-7. Hence, the present study was aimed to screen the soil bacteria for MK-7 production with intension of exploiting...
them for large scale production of MK-7.

2. Materials and methods

2.1. Collection of Soil samples and Isolation of Bacteria

Various soil samples were collected from different fields of the Gulbarga region i.e., Botanical garden, agricultural field, garden soil and were brought to the laboratory. The collected soils were serially diluted and plated on nutrient agar medium and incubated at 37°C for 24 h. To obtain a pure culture, the colonies were subcultured on the same medium by streaking method.

2.2. Screening of bacteria for MK-7 production

To detect the MK-7 production by the bacterial isolates, each isolate was inoculated in Erlenmeyer flask containing 100 ml cultivation media composed of (g/l) soya peptone (5g), yeast extract (5g), glycerol (5g) and K₂HPO₄ (0.06g) of pH 7. Thus, inoculated flasks were incubated at 37°C for 5 days in shaker incubator at 120 rpm [18] and thereafter, biomass was harvested for MK-7 extraction.

2.3. Extraction & Determination of MK-7

2.3.1. Extraction of Lipids

From the harvested biomass, lipid content was extracted by the modified Bligh and dyer method described by [19]. After 5 days of fermentation process, the broth were centrifuged at 10,000 rpm for 10 min at 22°C and the cell pellets were collected. Thus obtained pellet was mixed with 3ml of phosphate buffer, 7.5 ml of methanol and 3.75 ml of chloroform, shaken for few minutes and transferred to a separating funnel and allowed to stand for 2 h. Then 3.75 ml of chloroform and 3.75 ml of water were added, mixture was shaken well and allowed to separate the two layers for 1 h. The lower phase was collected and dried under the stream of nitrogen gas.

2.3.2. Identification of MK-7 by TLC

For the identification of presence of MK-7, the dried lipid extract was subjected for TLC along with standard MK-7. The dried lipid extract obtained was dissolved in minimal quantity of chloroform and was loaded on Silica gel GF₂₅₄ plates (Merck) in triplicates along with standard MK-7. The TLC was performed using Petroleum ether: Diethyl ether (85:15) as a solvent system [20]. The bands were observed under UV light at 254 nm and the Rf values were calculated.

3. Quantitative analysis of MK-7

The quantitative estimation of MK-7 was analyzed by using UV spectrophotometer as described by [21] wherein, from five days of fermentation broth, bacterial cell pellets were collected and subjected for the extraction of lipids. The collected lipids extract was analyzed for absorbance at 248 nm. The concentration of MK-7 was calculated with standard graph of MK-7.

4. Results and Discussion

In this study, a total of 140 bacterial isolates were isolated from various soil samples collected from the different regions of Gulbarga, Karnataka, India. The isolates were named as KLSG-1to KLSG-140. Screening studies about the production of MK-7 by these isolates revealed that, 60 isolates are capable to produce MK-7. Among these isolates, the five isolates viz. KLSG-9, KLSG-10, KLSG-5, KLSG-1 and KLSG-11 were noticed with the good yield (Table 1). Huang and Chen, reported that soil bacterium posses MK-7 as predominant menaquinones [22,23]. To our knowledge, this is the first attempt and report on the production of MK-7 by bacteria isolated from soil. Further, in the quantitative estimation the isolate KLSG-9 emerged as highest MK-7 producer. The isolate KLSG-9 identified as Gram positive bacilli (Figure 1). It has been previously reported that, menaquinones can be produced by Archaea and bacteria, such as Gram positive bacteria like flavobacteria and green sulfur bacteria [24]. All these reports stand with our findings of this investigation. The TLC results shown that, both sample and standard have same approximate Rf value of 0.7 (Figure 2). In TLC with silica gel, menaquinones migrates faster (Rf = 0.7) than ubiquinones (Rf = 0.4) [25]. The isolate KLSG-9 was able to produce comparatively highest yield of 48mg/L of MK-7 than other bacteria which have undertaken for investigation.

In the studies of [21] reported that the enhanced production of MK-7 by mutant strain BN-P15-11-1 and the yield was found to be 3.593 ± 0.107 mg/L whereas [26] noticed 28.26 ± 0.11mg/kg and 27.80 ± 0.06 mg/kg of MK-7 production by Bacillus subtilis (natto) under solid state fermentation using soy protein granules and nixtamalized corn grits. In similar type of study [27] reported 16.39µg MK-7 produced per gram of Glycine max and with Phaseolus vulgaris 31.35µg of MK-7 produced per gram of substrate by Bacillus subtilis NCIM 2708 after optimization of several fermentation parameters.

In Comparison of all these reports, the isolate KLSG-9 found to be quite competent and which could be

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Name of the strain</th>
<th>MK-7 yield (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>KLSG-1</td>
<td>14.0</td>
</tr>
<tr>
<td>2.</td>
<td>KLSG-5</td>
<td>20.0</td>
</tr>
<tr>
<td>3.</td>
<td>KLSG-9</td>
<td>48.0</td>
</tr>
<tr>
<td>4.</td>
<td>KLSG-10</td>
<td>21.6</td>
</tr>
<tr>
<td>5.</td>
<td>KLSG-11</td>
<td>10.0</td>
</tr>
</tbody>
</table>

Table 1: Production of MK-7 by the isolates.

Figure 1: Gram’s staining view of KLSG-9
resulted in considerable yield of MK-7 when it will be subjected for optimization. Hence, the isolate will be continued further with optimization and process development for the enhanced production of MK-7.

5. Conclusion
Various soil samples from different regions of Gulbarga were subjected for isolation and screening of bacteria for MK-7 production. Out of 140 bacteria isolated, only sixty isolates were found to produce MK-7. Further studies revealed that, the isolate KLSG-9 was capable to produce the considerable yield of MK-7. This investigation shows that, even soil bacteria are rich with MK-7 content. The present study suggest that, bacteria from soil can also be isolated rather than from any other sources and exploited for MK-7 production at large scale or industrial purpose.

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