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Millets based alternative sustainable cost-effective culture media for microbial growth

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Abstract

Millets are a rich source of starch, proteins, lipids, and other nutrients. This study aimed to assess whether millets can be used to formulate sustainable and economically viable culture media, thus potentially replacing the expensive traditional media used for growing microorganisms. Millet samples from Maharashtra, Rajasthan, and Tamil Nadu were assayed for their protein and lipid content. *Sorghum vulgare* (Jowar), *Pennisetum glaucum* (Bajra), and *Eleusine coracana* (Ragi) demonstrated high protein content ranging from 0.63–0.78 mg/ml. Using Thin Layer Chromatography, lipids extracted with hexane were fractionated into several bands and compared against standard fatty acids and cholesterol. *Panicum miliaceum* (Proso) and *Setaria italica* (Kang) showed the maximum levels of free fatty acids and cholesterol. Based on the protein, lipid, and nutrient content, millets were used in different compositions to formulate media for growing bacteria and fungi. A mixture of Ragi and Bajra, which serves as calcium and phosphate sources respectively, exhibited zones of phosphate solubilization, thus confirming its potential as an alternative to Pikovskaya medium, which is used to cultivate phosphate- solubilizing bacteria. A mixture of Varai and Rajgira, which serves as carbohydrate and protein sources respectively, showed luxurious growth of fungi, thus confirming its potential as an alternative to Sabouraud's Agar medium. Phosphate-solubilising bacteria are utilized in biofertilizer formation, thereby contributing to increased agricultural productivity. Besides proving a sustainable, environmentally friendly, and cost-effective alternative, the use of millets for media preparation can boost the agriculture sector and the economy of farmers as well.

Keywords

Millets; proteins; sustainable; culture media; biofertilizer; Pikovskaya

Introduction

Millets are climate-resilient crops. They can be easily grown in drought conditions due to their water-holding capacity. Millet is a product of sustainable farming that addresses world hunger. The International Crops Research Institute for Semi-Arid Tropics (ICRISAT) aims to increase millet production because of their high nutritional value. Being rich sources of starch, proteins, lipids, and micronutrients as well as cost-effective, millets can be used to prepare microbial growth culture media (1).

Pikovskaya medium is the primary medium used to cultivate phosphate-solubilizing microorganisms (PSM). Cultivation and isolation of PSM are routinely conducted by agroecologist researchers mainly to design suitable biofertilizers. PSM can make insoluble phosphate available to plants, thus enhancing soil phosphate utilization efficiency, promoting crop yields, and maintaining environmental sustainability (2, 3). Several media have been formulated in the past to screen for PSM. Multinational corporations are involved in developing biofertilizers using PSM and nitrogen-fixing microorganisms. This is projected to reduce fertilizer utilization by 30% and increase crop yield by 30% (4). Bashan *et al.* (5) reported tri-calcium phosphate as an appropriate universal selection factor for isolating PSM. Joe *et al.* (6) prepared the Soil Extract Calcium Phosphate (SECP) broth containing the following components: dextrose, $Ca_3(PO_4)_2$, KH_2PO_4, K_2HPO_4, and soil extract. Pikovskaya broth contains yeast extract, dextrose, agar, and chemicals such as calcium phosphate, ammonium sulphate, potassium chloride, magnesium, ferrous, and manganese sulphates. Compared to Pikovskaya broth, besides dextrose and buffering salts, the SECP broth eliminates the need for the addition of other chemicals.

Daniel A and Oboh E (7) formulated four different media sourced from millets and sweet potato, namely, Millet agar, Sweet Potato agar, Millet Glucose agar, and Sweet Potato Dextrose agar. They observed higher growth levels in millet agar compared to sweet potato agar, which could be due to the higher protein and fat content in millet, as bacteria proliferate more on high-protein foods. The fungal isolates *Aspergillus sp*. and *Rhizopus sp.* showed more growth on potato dextrose agar followed by sweet potato glucose agar, and the least growth was observed on millet agar.

Pikovskaya agar, used as a laboratory broth for growing PSM, costs 4000 Rs per 500 g. Sabouraud`s Dextrose agar, used to grow fungi, costs 7000 Rs per 250 g. Hence, the rationale behind this study was to find cost-effective, sustainable, alternative millet-based media to grow microorganisms. The use of chemicals in culture media leads to an environmental crisis (8). The natural resources of agar (i.e., *Gelidium sp., Gracillaria sp.* and *Pterocladia sp*.) are being over-exploited, prompting the scientific community to seek alternative sources (9). Thus, the aim of this study was to profile different millets for their nutritive values of protein and lipid content. Based on these findings, to formulate culture media containing millets in different combinations as per requirements to cultivate different microorganisms. These millet-based media can provide cost-effective and sustainable alternatives to Nutrient agar, Sabouraud's agar, and Pikovskaya agar. Millet-based media can be a low-cost solution, giving a thrust to agro-economic development without disturbing ecological and environmental balance.

Materials and Methods

Millets from Maharashtra (M), Rajasthan (R), and Tamil Nadu (T) were assessed to obtain their nutrient profiles. The millets used were Bajra (*Pennisetum glaucum*), Proso (*Panicum miliaceum* L), Foxtail/Kang (*Setaria italica*), Ragi (*Eleusine coracana*), Jowar (*Sorghum vulgare*), Pearl millet (*Pennisetum glaucum*), Varai (*Echinochloa frumentacae*) and Rajgira, a pseudomillet (*Amaranthus cruentus*).

Defatting of millet and sample preparation for protein estimation

In 1 g millet flour, 4 ml of n-hexane was added and incubated at room temperature for 1 hr. It was then centrifuged at 4000 rpm for 40 mins. The supernatant was decanted into another container. Approximately 1 ml of diethyl ether was added to the supernatant and further used for TLC analysis of lipids.

The pellet obtained was dissolved in 1 ml distilled water. It was allowed to stand at room temperature for 4 hr, and then centrifuged at 5000 rpm for 20 mins. The supernatant obtained was assayed for protein content by Lowry's method (10). Bovine serum albumin (0.1 mg/ml) was used as a standard. 1.0 ml of the sample and 5.0 ml of alkaline copper sulphate reagent were mixed and incubated at room temperature for 20 min. Then 1.0 ml of diluted Folin--Ciocalteu’s reagent was added. After 20 min of incubation at room temperature, absorbance was measured at 660 nm. The protein content in the sample was calculated from a standard curve for bovine serum albumin (11).

SDS-PAGE separation and identification of proteins in millets

The protein samples were subjected to SDS-PAGE separation using 10% separating gel and 5% stacking gel. A 100 µl sample was mixed with 100 µl Gel Loading Buffer. The protein markers and samples were loaded onto the gel and run at 50 V initially, and later at 100 V. The gel was stained with 0.1% Coomassie brilliant blue for 20 mins and then destained overnight using a destaining solution consisting of 40% methanol and 10% glacial acetic acid. The molecular weight of proteins was determined by comparison against a protein ladder containing 12 pre-stained proteins covering a range of molecular weights from 10 to 245 kDa (Pre-stained protein ladder-MBT092-10LN, HIMEDIA) (12).

Thin layer chromatography of lipids

Merck silica gel 60 F 254 HPTLC ready-made plates were used for separating lipids. The mobile phase used was petroleum ether: diethyl ether: glacial acetic acid in the ratio 80:40:2, and iodine was used as the detecting reagent (13).

Formulation of alternative media for Sabouraud's broth

Sabouraud’s broth generally contains peptone, tryptone, and dextrose. Hence, to substitute for the protein and carbohydrate source, Rajgira, being a rich source of proteins and lipids, and Varai, being a rich source of starch, were used to prepare an alternative millet-based media. 1 g of Rajgira powder, 2 g of Varai powder, 4 g of agar, and 100 ml of distilled water were mixed, autoclaved, and poured into sterilised empty petri plates. Sabouraud’s Agar plate was used as control.

Formulation of alternative media for Pikovskaya agar

Bajra is a rich source of phosphorus, and Ragi is a rich source of calcium (1). Other millets are rich sources of micronutrients. Hence, 1.3 g of Bajra powder, 1.2 g of Ragi powder, 1.2 g of Jowar powder, 0.03 g of Kang, 0.02 g of Proso, 0.3 g Foxtail, 3 g of agar, 0.05 g of yeast and 100 ml of water were mixed, autoclaved, and poured in sterilised empty petri plates. Pikovskaya Agar plate was used as control.

Study of the growth of microorganisms on millet-formulated media

Saccharomyces cerevisiae, *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* 113 3D, and soil suspension (1 gm soil in 2 ml saline) were inoculated on millet-formulated agar plates as tests, and on traditional Sabouraud's, Pikovskaya, and Nutrient agar plates as controls. Colonies were observed and counted after 24, 36, and 48 hrs of incubation at 37°C. Colony characteristics were noted, and desired colonies were subsequently screened and isolated.

Results and Discussion

Protein estimation

Protein content was calculated using the equation obtained from the standard graph as shown in Fig. 1. As illustrated in Table 1, Jowar (M), Jowar (R), Bajra (T), Bajra (R) and Ragi (M) showed high amounts of protein ranging from 0.63–0.78 mg/ml.

SDS-PAGE separation of proteins

Fractions in Bajra, Jowar, Ragi, and Foxtail were found to have a higher concentration of polypeptides with a molecular weight less than 10kDa. Almost every millet studied had a glutelin fraction around 21kDa to 24kDa. Bajra and Jowar have prolamin fractions around 13kDa. The significance of having small molecular weight proteins is that they are better utilized by the growing microorganisms. These millets are thus suitable to replace peptone and tryptone in traditional media, thus cutting down on the cost of the millet-based media.

Table 1. Protein content of millets (mg/ml/g of millet).

Sr No.	Sample	OD at 660 nm	Calculated protein content based on std equation y= 4.7511× Dilution factor 10 (mg/ml)	Sr No.	Sample	OD at 660 nm	Calculated protein content based on std equation y= 4.7511× Dilution factor 10 (mg/ml)
1	Jowar M (Sorghum)	0.36	**0.76**	8	Jowar R (Sorghum)	0.31	**0.65**
2	Jowar T (Sorghum)	0.23	0.48	9	Bajra T (Pearl millet)	0.37	**0.78**
3	Bajra R (Pearl millet)	0.31	**0.65**	10	Bajra M (Pearl millet)	0.11	0.23
4	Kang M (Foxtail)	0.11	0.23	11	Kang T (Foxtail)	0.13	0.27
5	Ragi T	0.25	0.53	12	Ragi M	0.30	**0.63**
6	Varai T	0.23	0.48	13	Varai M	0.07	0.15
7	Proso T	0.24	0.51	14	Rajgira M	0.16	0.34

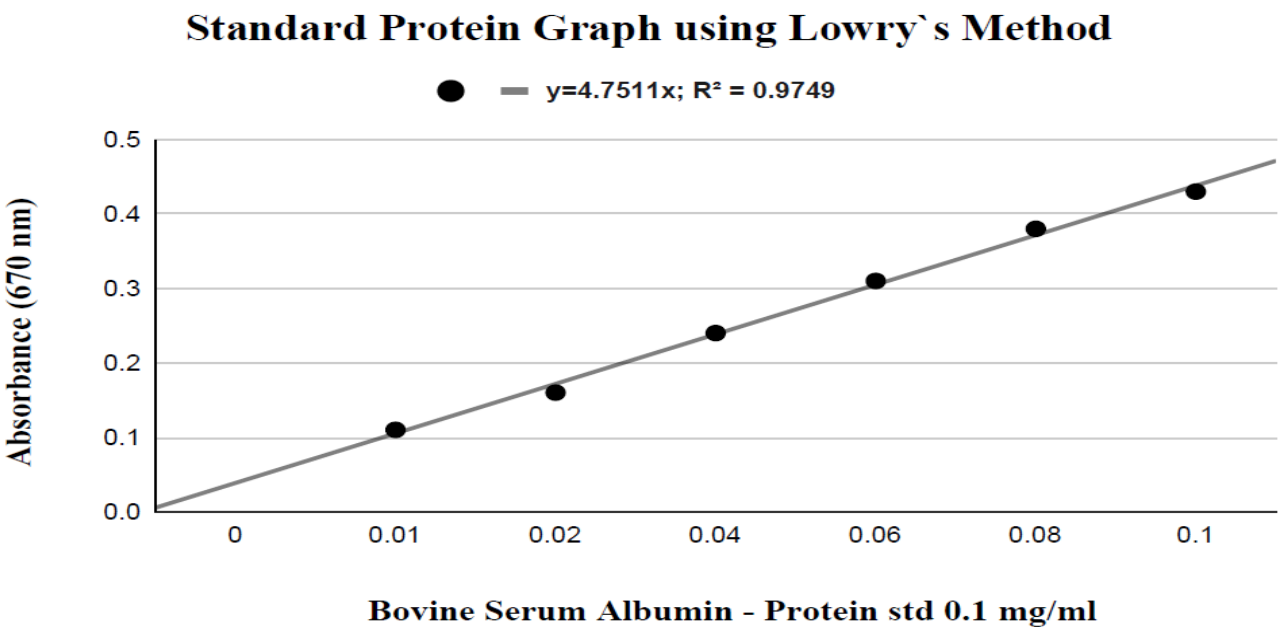

Fig. 1. Standard protein graph by Lowry`s method.

Thin layer chromatography of lipids

Proso and Kang showed free fatty acids and cholesterol, as shown in Fig. 2 and 3, respectively. Based on the literature review, the other bands that were separated can be identified as triglycerides and diacylglycerol (14).

Growth of microorganisms on millet-formulated media

Pikovskaya's media was used as the control, and alternative millet agar media were prepared and inoculated with soil suspension. Within 24 hr, millet agar showed exuberant growth, surpassing the control plate, with distinct colonies producing clear halo zones and exopolysaccharides, as shown in Fig. 4, 5, 6, and 7. Colonies were identified as white or light yellow, opaque, smooth, and some were transparent, belonging to *Azotobacter, Pseudomonas sp, Acinetobacter* and *Enterobacter sp*. (15). Millet-derived agar could yield a myriad of microbial diversity capable of solubilizing phosphate. Millet agar without yeast showed the highest exopolysaccharide secretion along with PSMs. Colonies were TMTC (too many to count), and exopolysaccharide secretion was identified by the presence of translucent colonies also exhibiting a clear zone, indicating phosphate solubilisation (16). Exopolysaccharides are compounds with high molecular weights and indirectly facilitates phosphate solubilisation in soil. Bacteria producing exopolysaccharides have a higher capacity to solubilize tricalcium phosphate (17).

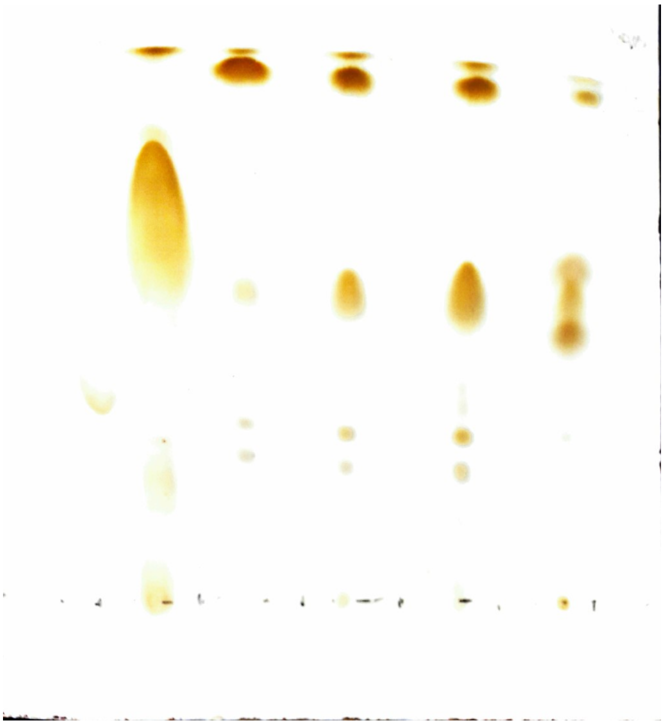

Fig. 2. Separation of lipids by Thin Layer Chromatography: Lane 1: Std Cholesterol Lane 2: Std Oleic Acid Lane 3: Bajra Lane 4: Proso Lane 5: Kang Lane 6: Varai.

Fig. 3. Separation of lipids by Thin Layer Chromatography: Lane 1: Std Cholesterol Lane 2: Std Oleic Acid Lane 3: Jowar Lane 4: Bajra Lane 5: Rajgira Lane 6: Ragi.

Fig. 4. Growth of colonies on millet agar slants.

Fig. 5. Fungal growth on millet-based media as an alternative to Sabouraud`s agar.

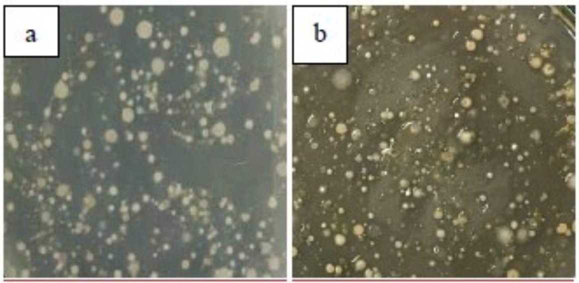

Fig. 6. Growth of varied colonies on millet-based media (b) as compared to control (a).

Millet agar proves to be superior to Pikovskaya broth in terms of bacterial growth, exopolysaccharide production, and phosphate solubilization. Luxuriant growth of phosphate-solubilizing microorganisms (PSM) on millet agar plates was observed compared to Pikovskaya agar, attributed to millets' natural and economical abundance of carbon, nitrogen, and B-vitamins. Pikovskaya media may have a limited diversity of PSM compared to millet-derived agar. Additionally, Pikovskaya media can cause irritation to the eyes, respiratory system, and skin, whereas millet-derived agar is safe and more effective. The Sabouraud's Agar control plate exhibited abundant fungal growth. Varai-Rajgira agar plates demonstrated the growth of various fungi, including *Aspergillus* and molds, identified based on their morphological characteristics such as conidial color and microscopy (18).

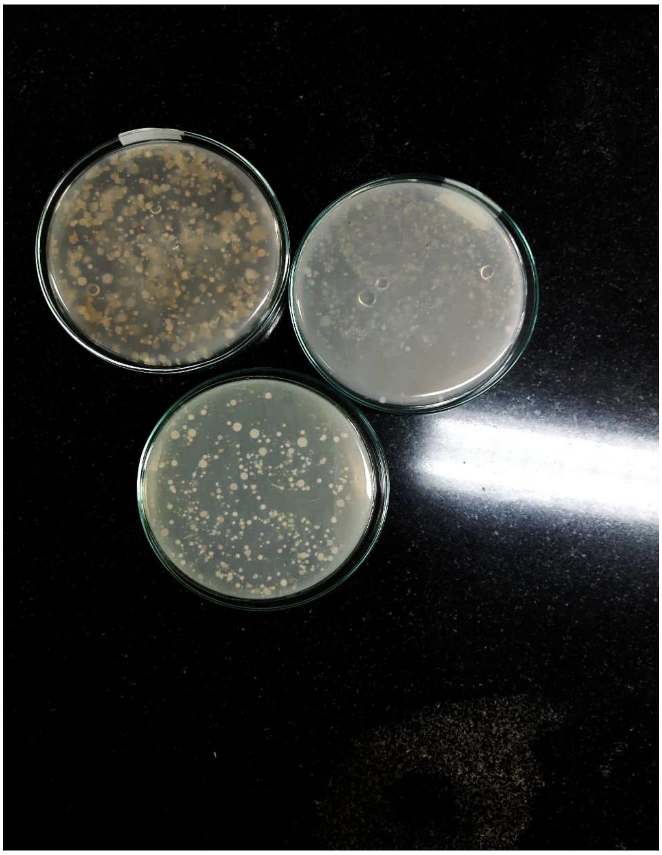

Fig. 7. Phosphate solubilising colonies on millet media with a clear halo and exopolysaccharide secretion.

Conclusion

Pikovskaya agar, priced at approximately Rs 4000 for 500 g, stands in stark contrast to its alternative, millet-derived agar, which costs only Rs 70 for the same quantity. Similarly, Sabouraud's dextrose agar, at around Rs 7000 for 250 g, is notably more expensive than Varai, which costs Rs 41 for 250 g. Millet-based culture media offer not only cost-effectiveness but also exhibit luxurious growth of phosphate solubilizers, complete with clear halo zones and exopolysaccharides within 24 hr. In contrast, traditional Pikovskaya agar requires 2 days for comparable results. The use of millets for culturing microorganisms presents a sustainable alternative. By cultivating phosphate solubilizers in laboratories using millet-based media, the demand for chemical phosphate fertilizers can be reduced, thereby mitigating their detrimental effects on the environment and decreasing costs. Consequently, this study suggests a potential increase in farmers' income, as the demand for millets rises not only for nutritional purposes but also for laboratory use. Synergistically this study is aligned with United Nations Sustainable Goal 11 of waste management and green economy, as well as with the declaration of the year 2023 as International Year of Millets. In future, high protein and rich amino acid profile of millets can replace the animal derived proteins such as meat or beef extract. Selective or enrichment media formulation for auxotrophs can be prepared using millets, and can replace expensive media like Mueller Hinton Agar for susceptibility testing, Mannitol Salt Agar, Tryptic Soy Agar, MRS Medium (deMan, Rogosa and Sharpe) for cultivating *Lactobacillus sp*, Chromogenic *Escherichia coli* Agar for rapid microbial testing and many more.

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Nil

Authors' contributions

JM, RP and KC put forth ideas and designed experiments. JM and RP performed the experiments. KC, JM and RP drafted the manuscript. KC conceived and coordinated the overall study. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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