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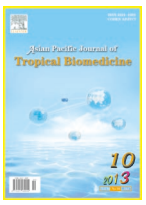

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Production of microbial medium from defatted brebra (*Milletia ferruginea*) seed flour to substitute commercial peptone agar

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PEER REVIEW

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Comments

This study found that defatted brebra seed flour with supplement and without supplement was by far better than the CPA. This study is a very good study that gives good clue for exploring important bio-products for developing new important growth media which is very cheap, inexpensive and easily available.
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ABSTRACT

Objective: To investigate and optimize microbial media that substitute peptone agar using brebra seed defatted flour.
Methods: Defatted process, inoculums preparation, evaluation of bacterial growth, preparation of cooked and hydrolyzed media and growth turbidity of tested bacteria were determined.
Results: Two percent defatted flour was found to be suitable concentration for the growth of pathogenic bacteria: *Escherichia coli* (ATCC 25922) (*E. coli*), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella* (NCTC 8385) and *Shigella flexneri* (ATCC 12022) (*S. flexneri*), while 3% defatted flour was suitable for *Staphylococcus aureus* (ATCC 25923) (*S. aureus*). *E. coli* (93±1) and *S. flexneri* (524±1) colony count were significantly ($P \leq 0.05$) greater in defatted flour without supplement than in supplemented medium. *E. coli* [$(3.72\times10^{9}\pm2)$ CFU/mL], *S. aureus* [$(7.4\times10^{9}\pm2)$ CFU/mL], *S. flexneri* [$(4.03\times10^{9}\pm2)$ CFU/mL] and *Salmonella* [$(2.37\times10^{9}\pm1)$ CFU/mL] in non-hydrolyzed sample were statistically ($P \leq 0.05$) greater than hydrolyzed one and commercial peptone agar. Colony count of *Salmonella* [$(4.55\times10^{9}\pm3)$ CFU/mL], *S. flexneri* [$(5.40\times10^{9}\pm3)$ CFU/mL] and *Lyesria moncytogenes* (ATCC 19116) [$(5.4\times10^{9}\pm3)$ CFU/mL] on raw defatted flour agar was significantly ($P \leq 0.05$) greater than cooked defatted flour and commercial peptone agar. Biomass of *E. coli*, *S. aureus*, *Salmonella* and *Enterococcus faecalis* in non-hydrolyzed defatted flour is highly increased over hydrolyzed defatted flour and commercial peptone broth.
Conclusions: The defatted flour agar was found to be better microbial media or comparable with peptone agar. The substances in it can serve as sources of carbon, nitrogen, vitamins and minerals that are essential to support the growth of microorganisms without any supplements. Currently, all supplements of peptone agar are very expensive in the market.

KEYWORDS

Colony counts, Commercial media, Defatted flour, Microbial media, Pathogenic bacteria, Peptone agar

1. Introduction

Preparation of media from cheap bio-products which are able to support and fulfill nutritional requirements for microbial growth is currently significant[1-3]. Currently, the culture media sold as pure dehydrated preparations have become very expensive in local market of developing nations and in most cases are not available. One major requirement of microbial media is a source of carbon[4] nitrogen, energy, sulphur, phosphorus and various

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minerals[5]. Culture media are liquid, semi solid and solid preparations widely used for the growth and identification of microorganisms in the laboratory. Most microorganisms are not capable of synthesizing all the amino acids, vitamins and other compounds essential for a living cell from simple materials such as inorganic nitrogen salts as long as they have a source of carbon and energy[6]. Although the substances used in the production of culture media are normally in the form readily absorbed by the microorganisms, complex organic substances drove from natural bioproducts are used for microbial growth[7]. To develop such important media, we have to search, evaluate, formulate and optimize media from bio-products from non-conventional plant sources[8,9]. Such type of bio-products may be served to substitute expensive media produced from different companies[10]. Of those expensive media, peptone agar is the one that currently used for different purposes in microbial culture. It is prepared from peptone, glucose, yeast extract and agar.

Peptones are defined as product of protein hydrolysis which are normally soluble in water and not heat coagulable. The nitrogen source for bacteria growth in media was obtained from biological substances such as urine, blood and other body fluids. Naegeli[11] was probably the first individual to use egg albumin as peptone source. Peptones, obtained by partial digestion of proteins, furnished organic nitrogen in more readily available forms. Microbial media was prepared from fish peptones drove from red hake (Urophycis cluss) and fishery by-products.

The nitrogen source is actually the most expensive component of bacterial growth substrates and at present it is obtained from plants such as legume seeds[12], dairy proteins such as casein or whey[13] and slaughterhouse waste. Fish hydrolysates and peptones are produced either by enzymatic digestion or acid hydrolysis of proteins[14,15]. Hydrolysis by the help of acid allows high yields; but this process cases to have high ash content which cannot be avoided through neutralization process.

Currently, the use of legumes as sources of protein hydrolysates in microbial culture media is common. Even now there is a need to explore this area for the production of comparatively standard culture media[16]. Peptones produced from legumes could serve as inexpensive nitrogen source for microbial culture and might improve microbial growth or product formation of some species of microorganisms. Among different species of legumes, brebra is a potential candidate to be served as a source of complex media in this investigation.

Brebra, *Milletia ferruginea* (Hochst.) Baker; is a tree that is endemic and widely distributed in the country, Ethiopia. It grows well in dry and moist lowland as well as wet and moist semi-highland agro climatic zones ranging from1000 to 2500 m above sea level[17]. It is widely applied to control insects such as pea aphid (*Achrythosiphon pisum*)[18] and fish poison in the country. Furthermore, the plant is extensively used as shade for coffee (*Coffea arabica*) in South part of Ethiopia[19] and it is also N_2 fixer, which serves to improve soil fertility of the degraded land[20] and leaf litter of this tree species to stimulate germination of coffee[21]. *Milletia ferruginea* seed oil has potential application for biodiesel and glycerol production[22]. Currently, tree can contribute to livestock production through provision of feed[23]. However, there is not any scientific finding of the use of defatted brebra seed flour as main substance for media production.

After oil extraction from brebra seed, there is a by-product, which is termed as defatted brebra seed flour. It is rich in protein content (48.5%)[24] and it was recommended to be used for microbial media production[25]. Thus, the defatted flour under investigation may be serving as a good substrate for microbial growth in general and can be used to substitute peptone agar in particular. The objective of this study is to investigate, formulate and optimize microbial media that serve as peptone agar from the plant under investigation.

2. Materials and Methods

2.1. Seed harvesting and dehulling process

The matured pods (pale yellow in colour) were collected in a ventilated sac and well dried until all seeds released from the pods. The released seeds from the pods were collected from the sac and dried by ovum over night at 30 °C to avoid moisture. The seed coat of the seeds was dehulled by roasted on pan.

2.2. Defatting process

The dehulled brebra seeds was milled with grinder (KIKA-WERKE-M20) and sieved through 250 μm sieve (ASTM EII, IMPACT, No-60). The flour was processed into two steps: defatting and protein extraction. The defatting process was carried out according to Wang *et al*[26]. Briefly, the brebra flour was defatted twice using hexane in flour to solvent ratio of 1:3 at a setting of 250 r/min in the lab stirrer for more than 2 h and filtered through vacuum funnel filtration. The defatted portion was air-dried over night, and milled again to powder form and sieved through 250 μm sieves and then packed in a bag and stored at 5 °C for further investigation.

2.3. Inoculums preparation

Escherichia coli (ATCC 25922) (*E. coli*), *Pseudomonas aeruginosa* (ATCC 27853) (*P. aeruginosa*), *Shigella dysenterae* (clinical isolate), *Staphylococcus aureus* (ATCC 25923) (*S. aureus*), *Shigella flexneri* (ATCC 12022) (*S. flexneri*), *Enterococcus faecalis* (29212) (*E. faecalis*), *Lyesria moncytogenes* (ATCC 19116) (*L. moncytogenes*), *Streptococcus pneumoniae* (ATCC 63) (*S. pneumoniae*) and *Salmonella* (NCTC 8385) were obtained from Ethiopian health and nutrition

research institution (EHNRI). Each bacterium was separately cultured on nutrient agar for 24 h[27,28]. Pure colonies were taken and inoculated to nutrient broth and incubated for 18 h and then 1 mL was serially diluted in sterile distilled water. From the 10-6 dilution, 0.1 mL was used to inoculate on agar medium prepared from defatted flour of brebra seed and commercial peptone agar (CPA). All tested microorganisms were grown at 37 ℃ on the two media (defatted brebra seed flour agar and petone agar) that have been autoclaved at 121 ℃ for 15 min. Bacterial colony counts were determined by the pour plate technique[29]. For each culture triplicate plates were prepared.

2.4. Evaluation of bacterial growth on defatted brebra seed flour agar (DBSFA) in comparison with peptone agar

Fastidious pathogenic bacteria were cultured on 2.5% of DBSFA supplemented with 1% glucose and 0.5% yeast extract, with only 1% glucose and without any supplement in 1 liter distilled water. In both cases, 1.5% agar was added. Standard (commercial) peptone agar (PA) medium was prepared to control purpose from 1% glucose, 0.4% peptone, 0.5% yeast extract and supplemented with 1.5% agar in 1 liter distilled water and the pH adjusted to 7.2 with 1 mol/L NaOH.

Two percent to five percent of DBSFA was used to determine the optimal amount of sample concentrations that required for each tested organisms. The same dilution (as mentioned above) was used to inoculate both peptone agar and DBSFA.

2.5. Cooked DBSFA

Fifty gram defatted brebra seed flour with 100 mL water was cooked for 30 min and then dried over night at 65 ℃ using oven. The cooked product was milled into powder and kept with plastic bag for further analysis. The powder was used as cooked DBSFA by using the same procedure mentioned above. Lastly, colonies of each plate were counted and the results were compared with that of uncooked DBSFA.

2.6. Protein hydrolysis

Defatted flour was hydrolyzed using pepsin-pancreatin enzyme systems. The method used by Sulieman *et al*[30,31] was slightly modified. About 80 g sample was suspended in 800 mL of 0.1 mol/L HCl containing 0.5 g pepsin and the mixture was incubated at 37 ℃ for 3 h, then neutralized with 0.5 mol/L NaOH and digested with 1.5 g pancreatin in 400 mL 0.2 mol/L phosphate buffer (pH 8.0), containing 0.005 mol/L sodium azide. The mixture was incubated at 37 ℃ for 24 h. The hydrolyzed protein was dried using lyophilizer (Bioblock scientific).

From hydrolyzed powder, 2.5% was taken and mixed with 1.5% agar and prepared by using the same procedure mentioned elsewhere in this study. As control, DBSFA and standard PA were also used by using the same procedure.

2.7. Determination of productivity ratio of the medium under investigation

Where it is necessary to determine the degree of growth productivity of microorganisms in the medium, the productivity was measured using productivity ratio (PR) method; PR=NS/NO, or

$$P_R = \frac{\text{No. of colonies on test} \times \text{dilution factor}}{\text{No. of colonies on control} \times \text{dilution factor}}$$

Where NS=total colony cont obtained on the tested culture medium and NO=total colony cont obtained on the defined reference culture medium[32]. This method was used in all test method when it was found to appropriate in this study. The type and amount of inoculum used was the same for both media and PR was calculated through counting the colonies on both test and control media.

2.8. Determination of growth turbidity of tested bacteria in prepared broth

From defatted brebra flour (non-hydrolyzed), 2.5% was dissolved in distilled water, and the pH adjusted to 7.0 with 1 mol/L NaOH without agar and the broth was processed by the procedure used for DBSFA. The same method was used for enzyme digested defatted brebra flour. Fresh two colonies of each test organisms were taken by wire loop from nutrient agar to inoculate the broths in triplicate. This was followed by incubation at 37 ℃ for 72 h on rotary shaker at 150 rpm in 100 mL culture flasks containing 50 mL of medium. Then, growth turbidity was measured from the broth culture with spectrophotometer at 540 nm for bacteria[33] at every 12 h interval. Un-inoculated peptone and DBSFB broth was used as blank. Growth turbidity of 0.4% standard (commercial) peptone broth enriched with 1% glucose and 0.5% yeast extract was used as control.

2.9. Determination of optimum pH for DBSFA medium

The DBSFA was prepared without pH adjustment and the other portion of the medium pH was adjusted in between 7 and 7.2. Both prepared media were autoclaved at 120 ℃ for 15 min and the result was evaluated.

2.10. Further culture medium treatment and production of MaCconkey agar using brebra seed defatted flour as nitrogen and carbon source

To produce quality microbial culture medium, the seed was finely powdered and defatted two times using hexane. Then, the solvent was recovered by Rota vapor and the defatted flour was again powdered using grinder (KIKA-WERKE-M20) and the moisture was removed by the help of oven at 60 ℃ for 25 h and then different pathogenic bacteria were cultured (using the same procedure used in the text before) to evaluate the quality of medium colour, appearance

and colony size in comparison with that of CPA.

One of MaCconkey agar is composed of peptone as source of nitrogen and carbon. The peptone portion of MaCconkey agar was substituted with 2 g defatted flour of brebra seed and mixed with 1 g lactose (Oxoid), 0.15 g bile salt (Oxoid), 0.03 neutral red (oxoid), 0.5 g NaCl, 0.001 g crystal violent, agar and 1.3 g agar (Oxoid) and mixed with 100 mL distilled water. Commercial MaCconkey agar (Oxoid), which composed of 2 g peptone, 1 g lactose, 0.15 g bile salt, 0.5 g NaCl, 0.03 g neutral red, 0.001 g crystal violent, 1.3 g agar and all were mixed with 100 mL distilled water, was used as control for comparison. In both cases, the pH was adjusted at 7.1±0.2 at 25 °C.

2.11. Statistical Analysis

All data were analyzed using SPSS version 16.0. Means and standard devaviations of the triplicates analysis were calculated using analysis of variance (ANOVA) to determine the significance differences between the means using Snedecor and Chochran method followed by Duncan's Multiple range test ($P<0.05$) when the F-test demonstrated significance. The statistically significant difference was defined as $P<0.05$.

3. Results

The potential of defatted brebra seed flour as media for the growth of pathogenic bacteria was evaluated (Table 1). Firstly, the effect of various concentrations (2% to 5%) of sample on the colony counts was investigated. Two percent defatted flour was found to be the most suitable concentration for the growth of fastidious pathogenic bacteria such as *E. coli*, *P. aeruginosa*, *Salmonella* and *S. flexneri* while 3% defatted flour was suitable for *S. aureus* species. These bacteria were selected as test organism since they need a special nutritious medium than normal flora. The colony counts number was decreased as concentration of sample increased above 3% (Table 1).

Table 1
The effect of different concentration of defatted brebra seed flour on some pathogenic bacteria growth (CFU/mL).

Test bacteria	Colony count of bacteria on defatted brebra seed flour of different concentration (CFU/mL)			
	2%	3%	4%	5%
E. coli	$2.46\times10^9\pm1^d$	$1.23\times10^9\pm1^c$	$2.11\times10^9\pm1^b$	$1.37\times10^9\pm1^a$
P. aeruginosa	$2.09\times10^9\pm1^d$	$1.69\times10^9\pm2^a$	$1.95\times10^9\pm2^c$	$1.85\times10^9\pm2^b$
S. aureus	$1.15\times10^9\pm2^a$	$1.57\times10^9\pm1^c$	$1.45\times10^9\pm1^b$	$1.08\times10^9\pm1^a$
S. flexneri	$1.46\times10^9\pm1^b$	$1.35\times10^9\pm1^a$	$1.36\times10^9\pm2^a$	$1.28\times10^9\pm3^a$
Salmonella	$2.87\times10^9\pm2^c$	$1.21\times10^9\pm3^b$	$6.80\times10^9\pm2^a$	$6.90\times10^9\pm2^a$

Values are means of triplicate determinations; Values within the same row followed by different superscripts are significantly different at ($P<0.05$).

For the growth of pathogenic bacteria medium prepared from defatted brebra seed flour with supplement and without supplement was by far better than the CPA (Oxoid) (Table 2). *E. coli* ($9.3\times10^9\pm1$) CFU/mL and *S. flexneri* ($5.24\times10^9\pm1$) CFU/mL colony count were significantly ($P\leq0.05$) greater in defatted flour without supplement than with supplemented medium. The colony counts in defatted flour with glucose supplement is statistically ($P\leq0.05$) greater than the medium with glucose and yeast extract supplement as well as the medium without any supplement for *Salmonella* ($5.68\times10^9\pm1$) CFU/mL and *S. aureus* ($2.64\times10^9\pm3$) CFU/mL. *L. moncytogenes* colony count ($5.70\times10^9\pm3$) CFU/mL on the medium supplemented with glucose and yeast extract was significantly ($P\leq0.05$) greater than other media in this study.

Table 2
Comparison of growth of some bacteria on defatted brebra seed flour agar against BGY, BG and CPA (CFU/mL).

Test bacteria	Colony count on defatted brebra seed flour with different supplements and without supplement (CFU/mL)			
	CPA	BGY**	BG***	B****
E. coli	$7.00\times10^9\pm1^b$	$5.50\times10^9\pm1^a$	$8.10\times10^9\pm1^c$	$9.30\times10^9\pm1^d$
Salmonella	$3.19\times10^9\pm1^a$	$4.17\times10^9\pm1^b$	$5.68\times10^9\pm1^d$	$4.55\times10^9\pm1^c$
S. flexneri	$6.70\times10^9\pm2^a$	$3.12\times10^9\pm1^b$	$4.68\times10^9\pm2^c$	$5.24\times10^9\pm1^d$
L. moncytogenes	$4.39\times10^9\pm1^a$	$5.70\times10^9\pm3^d$	$5.02\times10^9\pm2^b$	$5.40\times10^9\pm1^c$
S. aureus	$2.13\times10^9\pm1^d$	$1.92\times10^9\pm2^b$	$2.64\times10^9\pm3^c$	$1.70\times10^9\pm1^a$

BGY**=defatted flour supplemented with glucose and yeast extract, BG***=defatted flour supplemented with glucose, B****=defatted flour without supplement. The data was taken after 24 h of incubation but *Salmonella* and *S. aureus* was taken after 48 h of incubation.
Values are means of triplicate determinations; Values within the same row followed by different superscripts are significantly different at ($P<0.05$).

The colony counts of pathogenic bacteria on enzyme and non-enzyme treated defatted brebra seed flour agar was compared with CPA (Table 3). The colony count of *E. coli* ($3.72\times10^9\pm2$) CFU/mL, *S. aureus* ($7.40\times10^9\pm2$) CFU/mL), *S. flexneri* ($4.03\times10^9\pm2$) CFU/mL and *Salmonella* ($2.37\times10^9\pm1$) CFU/mL in non-hydrolyzed sample were statistically ($P\leq0.05$) greater than the colony count of the organisms in both hydrolyzed defatted flour agar and CPA. On the other hand, the colony counts of *S. pneumoniae* (6.72×10^9) CFU/mL and *E. faecalis* (1.57×10^9) CFU/mL were significantly ($P\leq0.05$) greater in hydrolyzed defatted flour agar than on non-hydrolyzed defatted flour agar and CPA.

Table 3
Comparison of colony counts of pathogenic bacteria on pepsin-pancreatic enzyme hydrolyzed and non-hydrolyzed defatted brebra seed flour agar in reference to CPA (CFU/mL).

Test bacteria	Non-hydrolyzed Sample (CFU/mL)	Hydrolyzed sample (CFU/mL)	Commercialpeptone agar(CFU/mL)
E. coli	$3.72\times10^9\pm2^c$	$2.21\times10^9\pm2^b$	$1.03\times10^9\pm1^a$
S. pneumoniae	$5.54\times10^9\pm3^b$	$6.72\times10^9\pm1^c$	$3.41\times10^9\pm2^a$
S. aureus	$7.40\times10^9\pm2^b$	$2.60\times10^9\pm1^a$	$7.20\times10^9\pm1^b$
S. flexneri	$4.03\times10^9\pm2^b$	$3.20\times10^9\pm1^a$	$3.90\times10^9\pm3^b$
Salmonella	$2.37\times10^9\pm1^c$	$1.74\times10^9\pm2^b$	$1.45\times10^9\pm1^a$
E. faecalis	$1.11\times10^9\pm2^b$	$1.57\times10^9\pm1^c$	$9.60\times10^9\pm2^a$

Values are means of triplicate determinations; Values within the same row followed by different superscripts are significantly different at ($P<0.05$).

The colony of bacteria after 24 h incubation on cooked and non-cooked defatted brebra seed flour agar against CPA is shown on Figure 1 and Table 4. Growth of some bacteria

on raw defatted flour agar (RDFA), cooked defatted flour agar (CDFA) and CPA was shown on Table 4. Colony count of *Salmonella* ($4.55\times10^9\pm3$) CFU/mL, *S. flexneri* ($5.40\times10^9\pm3$) CFU/mL and *L. moncytogenes* ($5.4\times10^9\pm3$) CFU/mL on RDFA were significantly ($P\leq0.05$) greater than on CDFA and CPA (Table 4 and Figure 1). The number of *E. coli* colonies ($1.12\times10^9\pm2$) CFU/mL grown on CDFA was statistically ($P\leq0.05$) greater in comparison with other prepared agar media but, the colony count of *S. aureus* ($2.64\times10^9\pm3$) CFU/mL was significantly ($P\leq0.05$) greater in CPA media than CDFA in this study.

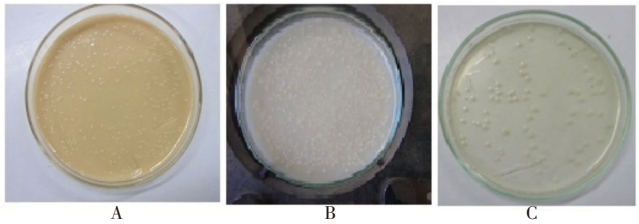

Figure 1. Colony of *S. flexneri* on differently treated defatted brebra seed flour agar and CPA.
A=from cooked DBFA, B=Non-cooked DBFA and C=Commercial peptone agar.

Table 4
Comparison of growth of some bacteria on RDFA, CDFA and CPA (CFU/mL).

Test bacteria	RDFA	CDFA	CPA
E. coli	$9.30\times10^9\pm1^b$	$1.12\times10^9\pm2^c$	$7.00\times10^9\pm1^a$
Salmonella	$4.55\times10^9\pm3^c$	$2.72\times10^9\pm2^a$	$3.19\times10^9\pm1^b$
S. flexneri	$4.55\times10^9\pm2^c$	$3.12\times10^9\pm3^b$	$6.70\times10^9\pm1^a$
L. moncytogenes	$5.40\times10^9\pm3^c$	$1.62\times10^9\pm2^a$	$4.39\times10^9\pm1^b$
S. aureus	$1.70\times10^9\pm2^a$	$2.30\times10^9\pm1^b$	$2.64\times10^9\pm3^c$

Values are means of triplicate determinations; Values within the same row followed by different superscripts are significantly different at ($P<0.05$).

Degree of growth productivity of microorganisms in the medium was measured using productivity ratio (PR) method[32]. This method is important to compare and contrast the productivity of culture media in the study with that of reference or standard commercial medium available in the market. In this study, the value of PR was greater than 1 in all test pathogenic bacteria except that of *S. aureus* (0.8) (Table 5). It implies that defatted brebra seed flour agar (BDFA) has a potential to be used for isolation, identification and sensitivity testing of different pathogenic microorganisms in the laboratory. However, further investigation is important for formulation and standardization of the medium for commercial use.

Table 5
Comparison of growth of some bacteria (colony count) on defatted brebra seed flour agar (BDFA) against CPA and productivity ratio (PR) of BDFA over CPA.

Test bacteria	Colony count on defatted brebra seed flour with different supplements and without supplement		
	CPA (CFU/mL)	BDFA (CFU/mL)	Productivity ratio
E. coli	$7.00\times10^9\pm1^b$	$9.30\times10^9\pm1^d$	1.33
Salmonella	$3.19\times10^9\pm1^a$	$4.55\times10^9\pm1^c$	1.43
S. flexneri	$6.70\times10^9\pm2^a$	$5.24\times10^9\pm1^d$	7.82
L. moncytogenes	$4.39\times10^9\pm1^a$	$5.40\times10^9\pm1^c$	1.23
S. aureus	$2.13\times10^9\pm1^d$	$1.70\times10^9\pm1^a$	0.80

The biomass of pathogenic bacteria in pepsin-pancreatic enzyme hydrolyzed, non-hydrolyzed defatted brebra seed flour broth and commercial peptone broth is shown on Table 6. The biomass of *E. coli*, *S. aureus*, *Salmonella* and *E. faecalis* at 24 and 48 h of incubation in non-hydrolyzed defatted brebra seed flour is highly increased over that of enzyme hydrolyzed defatted brebra seed flour and commercial peptone broth since optical density is proportional of biomass of pathogenic bacteria under investigation.

Table 6
Comparison of pepsin-pancreatic enzyme hydrolyzed and non-hydrolyzed defatted brebra seed flour broth in reference to commercial peptone broth (absorbance at 540 nm).

Test bacteria	Dilution factor	Hydrolyzed broth	Non-hydrolyzed broth	Commercial peptone broth
After 24 h of incubation		OD*	OD	OD
E. coli	20	0.1525	0.295	0.17
S. pneumoniae	20	0.331	0.284	0.2525
S. aureus	20	0.0325	0.589	0.0015
S. flexneri	20	0.364	0.586	0.303
Salmonella	20	0.1425	0.607	0.0455
E. faecalis	20	0.129	0.560	0.033
After 48 h of incubation				
E. coli	20	0.209	0.285	0.160
S. pneumoniae	20	0.338	0.511	0.357
S. aureu	20	0.160	0.553	0.002
S. flexneri	20	0.663	0.601	0.342
Salmonella	20	0.251	0.653	0.300
E. faecalis	20	0.136	0.634	0.026
After 72 h of incubation				
E. coli	20	0.600	0.236	0.157
S. pneumoniae	20	0.390	0.366	0.360
S. aureus	20	0.285	0.713	0.133
S. flexneri	20	0.732	0.489	0.221
Salmonella	20	0.424	0.480	0.337
E. faecalis	20	0.318	0.588	0.036

OD*=Optical density.

The pH of the agar and broth medium of defatted brebra seed flour should be adjusted from 7 to 7.2. Otherwise the entire medium substances will precipitate at pH value less than 6.5 (Figure 2).

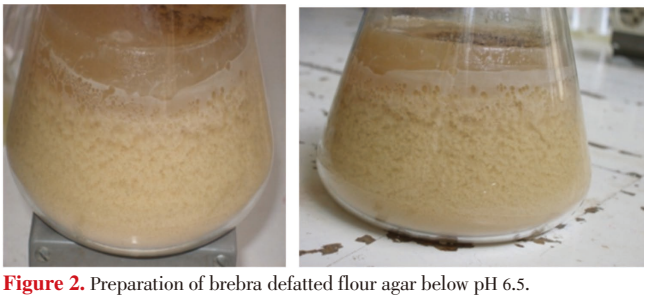

Figure 2. Preparation of brebra defatted flour agar below pH 6.5.

After further culture medium treatment as mentioned in the method, the medium appearance and colony characteristics of tested pathogenic bacteria grown on defatted flour were compared and contrast with that of the

medium appearance and colony characteristics of the same bacteria cultured on CPA (Figure 3). Almost the qualities of medium and colony characteristics of tested bacteria grown on defatted flour were in line and comparable with that of peptone agar.

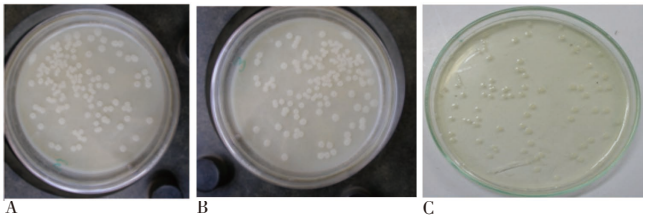

Figure 3. Colony of *S. flexneri* on further treated defatted brebra flour agar (A &B) and CPA (C).

The colony colour and other morphological qualities of *S. flexneri* grown on commercial MaCconkey agar (Oxoid) (A) and MaCconkey agar was made from defatted brebra flour as peptone substitute (B) (Figure 4). The colony colour of *S. flexneri* grown on commercial MaCconkey agar (Oxoid) (A) was whiter than grown on MaCconkey agar made from defatted brebra flour as peptone substitute (B). However, the colony size of *S. flexneri* grown on MaCconkey agar made from defatted brebra flour as peptone substitute (B) was much larger than grown on commercial MaCconkey agar (Oxoid) (A).

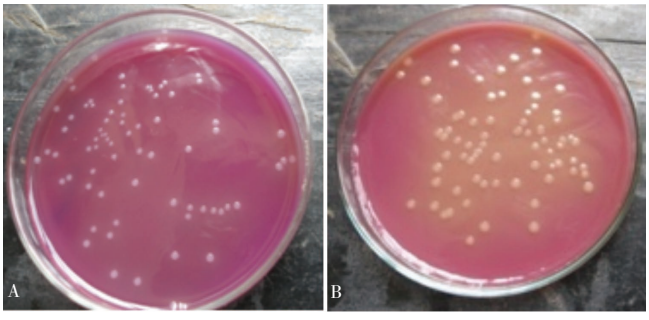

Figure 4. Colony of *S. flexneri* on commercial MaCconkey agar (Oxoid) (A) and MaCconkey agar was made from defatted brebra flour as peptone substitute (B).

4. Discussion

The main objective of the study was to analyze the microbial medium formulated from brebra defatted flour and compare it with a commercial medium like peptone agar commonly used in microbiological cultivation. The prepared medium was found to support growth of the tested bacteria, indicating that the prepared medium has huge potential to substitute CPA and other related commercial media widely used in cultivation of bacteria.

The main chemical composition of defatted sample under this investigation was determined[24]. Clearly, it contains high quantity of essential substances such as minerals, amino acids, carbohydrate and others required in a bacteriological media for microbial growth. These important substances that serve as sources of carbon, nitrogen and minerals are essential to support the growth of microorganisms without any supplement. Thus, the chemical composition of defatted brebra flour is consistent with different investigations on nutritional composition of various sources[34].

Two percent of defatted brebra seed flour agar was the concentration observed to have maximum colony growth and number for most pathogenic bacteria. The colony counts number was decreased as concentration of sample increased above 3%. The high concentration of the substrate may have inhibiting effect due to the high biochemical oxygen demand load of defatted brebra seed flour.

According to this finding, medium without supplement is far better than CPA in both colony counts. The colony counts of pathogenic microorganisms grown on defatted flour agar with and without supplement were also better than the same organisms grown on CPA. The possible reason is that defatted seed flour of brebra may contain all required nutritional values for the growth of fastidious pathogenic bacteria. In the same manner, the colony count of organism on non-hydrolyzed sample is better than hydrolyzed sample in this study. Thus, digestion of defatted flour by enzymes is not so important for medium preparation from the sample under this investigation. On the other hand, cooking is not important to increase the colony count of tested organisms in this finding. The assumption of cooking was to assess the effect of anti-nutritional and enzyme inhibitory factors on the growth of pathogenic bacteria since these organisms are more sensitive to any changed physico-chemical characteristics of the medium. In conclusion, the growth biomass of pathogenic bacteria is greater in non-hydrolyzed defatted flour than hydrolyzed one and commercial peptone broth. Thus, further treatment with enzyme is not necessarily important for this medium preparation.

Commercial MaCconkey agar (Oxoid) is composed of 2 g peptone, 1 g lactose, 0.15 g bile salt, 0.5 g NaCl, 0.03 g neutral red, 0.001 g crystal violent, 1.3 g agar. Peptone is the nitrogen and vitamin source in MacConkey Agar. Lactose is the fermentable carbohydrate. Fermentation of lactose causes to drop pH around the colony and change the colour of pH indicator, which is called neutral red in this case and bile precipitation. Bile salts mixture and crystal violet are used to inhibit Gram-positive cocci and only vabor Gram-negative organisms grow in MaCconkey agar. Sodium chloride is applied to maintain the osmotic environment. Agar is, as it is known, the solidifying agent. In this study, brebra defatted flour was used as peptone and the rest ingredient of MaCconkey agar included in proportion with commercial MaCconkey agar. The colony colour of *S. flexneri* was almost similar with commercial MaCconkey agar and the size of the colonies was better than colonies grown on commercial MaCconkey agar. However, it needs further investigation to produce medium with consistent basic characteristics.

In summary, to prepare CPA, yeast extract as growth promoters or source of vitamins, sugars as carbon source and peptones as nitrogen source should be used to allow the growth of bacteria. All these supplements are currently

very expensive in the market particularly in resource poor nations, including Ethiopia. The defatted brebra seed flour under this investigation was found to be rich in nutrition content. These substances can serve as sources of carbon, nitrogen and minerals that are essential to support the growth of microorganisms without any supplement as pointed out above. Defatted brebra seed flour has huge potential to be used as raw material for the formulation of different microbial media such as Muller Hinton agar, nutrient agar, MaCconkey agar and so forth.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

This study found that defatted brebra seed flour with supplement and without supplement was by far better than the CPA. This study is a very good study that gives good clue for exploring important bio-products for developing new important growth media which is very cheap, inexpensive and easily available.

Research frontiers

This study analysed and compared medium formulated from brebra defatted flour with a commercial medium of peptone agar. For the growth of pathogenic bacteria medium prepared from defatted brebra seed flour with supplement and without supplement was by far better than the CPA. Growth of organism was even greater in defatted brebra flour without supplement and non enzyme hydrolyzed than with supplemented enzyme hydrolyzed medium. The defatted brebra seed flour under this investigation was found to be rich in nutrition content. These substances can serve as sources of carbon, nitrogen and minerals that are essential to support the growth of microorganisms without any supplement.

Related reports

The main chemical composition of defatted sample under this investigation was determined (Andualem and Gessesse, 2012). Clearly, it contains high quantity of essential substances such as minerals, amino acids, carbohydrate and others required in a bacteriological media for microbial growth. These important substances that serve as sources of carbon, nitrogen and minerals are essential to support the growth of microorganisms without any supplement. Thus, the chemical composition of defatted brebra flour is consistent with different investigations on nutritional composition of various sources (Allexiou *et al.* 1980; Baden and Kubilus, 1983; Kurbanoglu and Algur, 2002; Yousuf *et al.* 2007).

Innovations & breakthroughs

The defatted brebra seed flour under this investigation was found to be rich in nutrition content. These substances can serve as sources of carbon, nitrogen and minerals that are essential to support the growth of microorganisms without any supplement as pointed out above. Defatted brebra seed flour has huge potential to be used as raw material for the formulation of different microbial media such as Muller Hinton agar, nutrient agar, MaCconkey agar and so forth.

Applications

This study may be very important and applicable for substituting some of the media like peptone agar. The content of the defatted brebra seed flour under investigation may be important for further investigation wether this content is also important for growing other fastidious organisms that can not be easily grown on general purpose media.

Peer review

This study found that defatted brebra seed flour with supplement and without supplement was by far better than the CPA. This study is a very good study that gives good clue for exploring important bio-products for developing new important growth media which is very cheap, inexpensive and easily available.

References

[1] Marikar FM. Cocos nucifera's watery endosperm as a potential culture medium for fungal growth. *Micol Aplicada Int* 2009; **21**: 63-66.

[2] Tharmila S, Jeyaseelan EC, Thavaranjit AC. Preliminary screening of alternative culture media for the growth of some selected fungi. *App Sci Res* 2011; **3**(3): 389-393.

[3] Deivanayaki M, Antony IP. Alternative vegetable nutrient source for microbial growth. *Int J Biosci* 2012; **2**(5): 47-51.

[4] Farhana MS, Biviand M, Khairulmazmi A. Effect of carbon sources on bacterial production of metabolites against *Fusarium oxysporum* and *Colletotrichum gloeosporioides*. *Int J Agr Biol* 2011; **13**(1): 1-8.

[5] Harvey RA, Champe PC, Fisher BD. Lippincott's illustrated *reviews: microbiology*. 2nd ed. Philadelphia: Lippincott Williams & Wilkins; 2007.

[6] Seeley HW, Van Denmark PJ. *Microbes in action a laboratory manual of microbiology*. 3rd ed. USA: W.H. Freeman; 1981.

[7] Kastner M, Brewer-Jammali M, Mahroe B. Enumeration and characterization of the soil micro flora from hydrocarbon contaminated soil sites able to mineralize polycyclic aromatic hydrocarbon (PAH). *Appl Microbiol Biotechnol* 1994; **41**: 267-273.

[8] Gutiérrez-Rojas I, Torres-Geraldo AB, Moreno-Sarmiento N. Optimising carbon and nitrogen sources for *Azotobacter chroococcum* growth Ivonne. *Afr J Biotech* 2011; **10**(15): 2951-2958.

[9] Famurewa O, David OM. Formulation and evaluation of dehydrated microbiological media from avocado pea (*Peasea Americana* Cmill). *Res J Microb* 2008; **3**(5): 326-330.

[10] Arulanantham R, Pathmanathan S, Ravimannan N, Niranjan K. Alternative culture media for bacterial growth using different formulation of protein sources. *J Nat Prod Plant Resour* 2012; **2**(6): 697-700.

[11] Naegeli C. *Peptone Akad*. Munich; 1880.

[12] Annan-Prah A, Akorli SY, Sedofia KB. Growth and cultural characteristics of selected bacteria on cowpea agar (*Vigna unguiculata*). *Afr J Microb Res* 2010; **4**(23): 2626-2628.

[13] Lund B, Norddahl B, Ahring B. Production of lactic acid from whey using hydrolysed whey protein as nitrogen source. *Biotechnol Lett* 1992; **14**(9): 851-856.

[14] Dufosse L, De La Broisse D, Guerard F. Fish protein hydrolysates as nitrogen source for microbial growth and metabolite production. *Recent Res Dev Microbiol* 1997; **1**: 365-381.

[15] Girón-Calle J, Alaiz M, Vioque J. Effect of chickpea protein hydrolysates on cell proliferation and *in vitro* bioavailability. *Food Res Int* 2010; **43**(5): 1365-1370.

[16] Nigam PS, Pandey A. *Biotechnology for agro-industrial residues utilization: utilization of agro-residues*. Netherlands: Springer Science Business Media B.V.; 2009.

[17] Bekel-Tesemma A, Birnie A. Tengnas B. *Useful trees and shubs of Ethiopia; Identification, propagation and management for agricultural and pastoral communities*. Sweden: Regional soil Conservation Unit, Swedish International Development Authority; 1993, p. 356.

[18] Hora AZ, Seyoum E, Mulatu B. *Application of Millettia ferruginea in pest management: effects of crude extracts of Millettia ferruginea seed powder in solvents of different polarity on Acrythosiphon pisum (Paperback)*. Germany: LAP Lambert Academic Publishing; 2012.

[19] Muleta D. *Microbial inputs in coffee (Coffea arabica L.) production systems, Southwestern Ethiopia*. Uppsala: Acta Universitatis agriculturae Sueciae; 2007.

[20] MacLachan M, Eshetie G, Fentahum T. *Manual of high land Ethiopian trees* (English/Amharic version). Addis Ababa: SIM Forestry Study project; 2001.

[21] Girma Y, Wolka K. Effects of *Albizia gummifera*, *Millettia ferruginea* and *Cordia africana* Leaf litter on the germination of *Coffea arabica* L. Seed. *Int J Agri Res* 2012; **7**(6): 315-323.

[22] Andualem B, Gessesse G. Production and characterization of biodiesel from brebra (*Milletia ferruginea*) seed non-edible oil. *Biotech* 2012; **11**(4): 217-224.

[23] Alemu B, Animut G, Tolera A. *Millettia ferruginea*: An endemic legume tree as forage for ruminants in southern and northwestern Ethiopia. *Livestock Res Rural Dev* 2013; **25**(3): 44.

[24] Andualem B, Gessesse A. *Biotechnological application of brebra (Millettia ferruginea) (Hochst) Baker seed for the production of biodiesel and protein*. Addis Ababa: Addis Ababa University; 2010.

[25] Andualem B, Gessesse G. Methods for refining of brebra (*Millettia ferruginea*) oil for the production of biodiesel. *World App Sci J* 2012; **17**(3): 407-413.

[26] Wagner PR. *Sol-gel immobilization of lipase for the hydrolysis of soybean oil*. USA: University of Nebraska-Lincoln; 1999.

[27] Andualem B. Synergistic antimicrobial effect of Tenegn honey (*Trigona iridipennis*) and garlic against standard and clinical pathogenic bacterial isolates. *Int J Micro Res* 2013; **4**(1): 16-22.

[28] Andualem B. The combined antibacterial activity of stingless bee (*Apis mellipodae*) honey and garlic (*Allium sativum*) extracts against standard and clinical pathogenic bacteria. *Asian Pac J Trop Biomed* 2013; **3**: in press.

[29] Reasoner DJ, Geldreich EE. A new medium for the enumeration and subculture of bacteria from potato water. *Appl Environ Microbiol* 1985; **49**: 1-7.

[30] Sulieman MA, Hassan AB, Osman GA, El Tyeb MM, El Khalil EA, El Tinay AH, et al. Changes in total protein digestibility, fractions content and structure during cooking of lentil cultivars. *Pakistan J Nutr* 2008; **7**(6): 801-805.

[31] Mune MA, Minka SR, Mbome IL, Etoa FX. Nutritional potential of Bambara bean protein concentrate. *Pakistan J Nutr* 2011; **10**(2): 112-119.

[32] Culture Media SIG. *Guideline for assuring quality of medical microbiological culture media*. 2012. [Online] Available from: http://www.theasm.org.au/assets/ASM/Guidelines-for-the-Quality-Assurance-of-Medical-Microbiological-culture-media-2nd-edition-July-2012.pdf. [Accessed on 20 August 2013]

[33] Uzeh RE, Akinola, Olatope SO. Production of peptone from soya beans (*Glycine max* L merr) and African locust beans (*Parkia biglobosa*). *Afr J Biotechnol* 2006; **5**(18): 1684-1686.

[34] Yousuf AA, Mofio BM, Ahmed AB. Nutrient contents of pride of Barbados (*Caesalpinia pulcherrima* Linn.) seeds. *Pakistan J Nutr* 2007; **6**: 117-121.