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Research article

STUDY ON PROBIOTIC POTENTIAL AND LABORATORY SCALE PRODUCTION OF LACTIC ACID BACTERIA.

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ABSTRACT: Lactic acid bacteria were isolated from dairy food. They were identified on the basis of their morphological, cultural and biochemical characterastics.The cell free supernatant of lactic acid bacteria were able to inhibit the growth of *E.coli, Klebsiella aerogens, Salmonella spp. , S.aureus, P.mirabilies and Ps .aeruginosa.* The probiotic properties of isolate of lactic acid bacteria (LAB-VI) were investigated. The LAB-VI was susceptible to antibiotics like ampicillin, gentamycin, ciprofloxacin, ofloxacin and streptomycin. Maximum biomass of LAB – VI was obtained at pH: 5, inoculum size 4% v/v & incubation period 48 hrs. Efficacy of coconut extract medium (Type I and Type II) on growth, lactic acid and hydrogen peroxide production by LAB-VI was studied using laboratory bioreactor in batch fermentation. The type II coconut extract medium gave higher biomass yield than type I (coconut extract medium). The biomass production from MRS was more than coconut extract medium. The formulated coconut extract medium (Type II) should be used to substitute MRS, since the substrate is economical, readily available and reduce quantity of the expensive supplements. The bacteriocin activity of the bacteriocin from fermented Type I & Type II coconut extract media were 1:6 & 1:8 respectively .The bacteriocin activity was tested against *Klebsiella aerogens*

Keywords: Batch fermentation, Coconut extract medium, Lactic acid bacteria, Bacteriocin.

INTRODUCTION

Probiotics are live microorganisms, which when administered in adequate amounts; confer health benefit on the host. Probiotics protect host by production of inhibitory substances, blocking of adhesion sites, and competition for nutrients, stimulation of immunity and degradation of toxin receptor. The probiotics can be bacteria, molds or yeasts. Most probiotics are bacteria. Commonly used bacterial probiotics include, *Lactobacillus* Species, *Lactococcus* species, *Streptococcus* species, *Pedicococcus species*, *Bifidobacterium species, E.coli* and *Bacillus specie*s. Safety aspects of probiotic bacteria include, strains for human use are preferred to be of human origin, they are isolated from healthy human gastrointestinal tract, they have to be non-pathogenic, they have to no history of relationship with diseases like, infective endocarditic or gastrointestinal tract disorders and they do not deconjugate bile salts. The clinical applications of probiotics are management of lactose intolerance, improving immune system ,prevention of colon cancer, reduction of cholesterol and triacylglycerol plasma concentrations (weak evidence), lowering blood pressure, reducing inflammation, reduction of allergic symptoms, beneficial effects on mineral metabolism particularly bone density and stability, reduction of *Helicobacter pylori* infection, suppression of pathogenic microorganisms (antimicrobial effect)and prevention of osteoporosis. In order to be able to exert its beneficial effects, a successful potential probiotic strain is expected to have a number of desirable properties like, human origin for human usage, acid and bile tolerance, adhesion to mucosal surface, safe for food and clinical use and good technological properties.. Probiotics include probiotic drugs, probiotic foods, direct- fed microbial and designer probiotics (Sander, et al., 2009 and Guarner, F., 2011). Probiotic fortified foods are fermented foods which include: Cheese, Yoghurt, Meso, Temph, Soysausce and Ice Cream Beverages and Chocolates. Indian probiotic market is valued at $2 million as per 2010 estimates and it is poised to quadruple by 2015 due to the advent of Indian and multinational companies coming in to the fray **.** Current players In Indian probiotic market are, Yakult Danone India, Amul, Nestle and Mother Dairy Pvt. Ltd. Probiotic products are contributing to 15% of the turnover of their fresh dairy products(Raja, et al.,2011). The objectives of this investigation were isolation and identification of lactic acid bacteria from dairy products, determination of probiotic properties of lactic acid bacteria, optimization of growth conditions for probiotic production and testing of efficacy of coconut extract media in probiotic production.

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MATERIALS AND METHODS

Isolation and Identification of LAB

Curd, cheese &butter samples were procured from different dairies of Gondia City (Maharashtra). Lactic acid bacteria were isolated by standard procedure (Punyart, et al., 2008).Pure cultures were identified according to their morphological, cultural and biochemical characteristics by the procedures described in Bergey's Manual (Krieg, et al., 1984).The identification tests used were Gram reaction, motility test, endospore formation, production of catalase and oxidase, Hugh - Leifsons test in O/F medium and fermentation of sugars.

Growth inhibition activity of LAB isolates

Growth inhibition activity of LAB isolate against test organisms (*E.coli, Klebsiella aerogens, Salmonella spp., S.aureus, P.mirabilies, Ps .aeruginosa*) was evaluated by agar well diffusion method. The inhibition zone was measured in millimeters by zone reader (Schillinger, et al., 1989).

Determination of Probiotic Properties of LAB-VI isolate

For the determination of probiotic potential of LAB-VI isolate, the major selection criteria chosen include: - resistance to low pH, tolerance against bile salt & antibiotic resistance.

Antibiotic Resistance of LAB

The antibiotic resistance of LAB-VI isolate was assessed by agar disc diffusion method using antibiotic discs of Hi-Media Laboratories Pvt. Ltd. Mumbai, India on Mueller-Hinton Agar plate (Bauer, et al., 1966). Resistance was assessed against ampicillin (l0mcg), gentamycin (l0mcg), ciprofloxacin (5mcg), ofloxacin (5mcg) and streptomycin (10mcg). The diameter of growth inhibition zones were measured after incubation for 48 hours in aerobic condition & compared with those in interpretative standard chart. The results were reported as resistant & susceptible.

Resistance to Low pH

Resistance to Low pH (pH: 3) is often used in vitro assays to determine the resistance to stomach pH. Because the foods are staying during 3 hrs, this time limit was taken into account (Prasad, et al., 1998). Acid tolerance of isolates was determined as per the procedure described earlier but with some modifications (Hyronimus, et al., 2008).

Tolerance against Bile

The strain resistant to low pH was screened for their ability to tolerate the bile salt. Although the bile concentration of the human gastro intestinal tract varies, the mean intestinal bile concentration is believed to be 0.3% w/v and the staying time is suggested to be 4 hrs. The bile resistance of the isolate was evaluated by earlier method with some modifications (Arihara, e al., 1998).

Optimization of growth conditions for good growth of Lactic Acid Bacteria

The effect of growth conditions on the LAB-VI isolate, with good probiotic properties i.e. strong antagonistic activity, resistance to antibiotics, survival at low pH & bile salt tolerance, was studied.

The inoculum was prepared by inoculation of a loopful refrigerated stock in 10ml MRS broth (pH 6.5) & incubating for 24 hrs at 37^0C aerobically.

To study the effect of pH, the sterile fermentation medium i.e. De Man, Rogosa and Sharpe broth (MRS broth) was used .The pH of the medium was adjusted to 2 to 8 before sterilization. 24 hrs old 1% v/v inoculum was used to inoculate series of flasks, each with 100ml fermentation medium. Flasks were incubated aerobically at 37^0C for 24 hrs in stationary condition. After 24 hrs, optical density of fermented broth was measured at 600 nm.

To study the effect of inoculum size, the sterile fermentation medium (MRS broth with pH 5.0) was used.24 hrs old 1 to 6% v/v inoculum was used to inoculate series of flasks, each with 100 ml fermentation medium. Flasks were incubated aerobically at 37^0C for 24 hrs in stationary condition. After 24 hrs, optical density of fermented broth was measured at 600 nm.

To study the effect of incubation period, the sterile fermentation medium (MRS broth) was used. The pH of the medium was adjusted to 5.0 before sterilization, 4% v/v 24 hrs old inoculum was used to inoculate series of flasks, each with 100 ml fermentation medium. Flasks were incubated aerobically at 37^0C up to 120 hrs in stationary condition. After incubation period of 24,48,72,96 and 120 hrs, optical density of fermented broth was measured at 600 nm. The OD of culture was converted to dry cell mass through a linear correlation standard curve. 1 OD_{600} was almost equivalent to 0.3 g L^{-1}(Malek, et al., 2011).

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Bioreactor Cultivation

Cultivation of LAB-VI isolate, under optimized conditions in coconut extract medium (Type I and Type II) was carried out in 5 – L stirred tank bioreactor with working volume 4 – L.The composition of type I and type II coconut extract media were mentioned in table 1 & 2 respectively. The bioreactor is equipped with pH probe & agitator. The pH of the medium was adjusted to 5.0 before inoculation. During the experiment, temperature & the optimum speed were controlled at 37^0C & 100 rpm respectively. The total time of fermentation was 24 hrs. Samples were taken at 0 hrs, 4 hrs, 8 hrs and 24 hrs and analyzed immediately for cell mass, pH change, glucose concentration (Miller, et al., 1959), lactic acid and hydrogen peroxide concentration (A.O.A.C., 1990).

Table 1: Type I Coconut extract medium

S.N.	Ingredients	Quantity
1	Ammonium Citrate	2 gm
2	Sodium Acetate	5 gm
3	Magnesium Sulphate	10 gm
4	Manganese Sulphate	0.05 gm
5	Dipotassium Phosphate	2 gm
6	Coconut extract	1000 ml
	$_p$H - 5.0	

Table 2: Type II Coconut extract medium

S.N.	Ingredients	Quantity
1	Protease Peptone	5 gm
2	Beef Extract	5 gm
3	Yeast Extract	2.5 gm
4	Glucose	10 gm
5	Coconut extract	1000 ml
	$_p$H - 5.0	

Extraction and Determination of Bacteriocin Activity.

For extraction of bacteriocin, a cell free solution was obtained by centrifugation (6000 rpm for 20 min, 4^0C) and cell free solution was adjusted to pH 6.5 by means of 10 N NaOH to exclude antimicrobial effect of organic acids. The bacteriocin activity was quantitated by spotting 20 micro liter aliquots of twofold serial dilutions of culture supernatant that was spotted onto the surface of MRS agar inoculated with 0.5 ml *Klebsiella aerogens* (Oh et.al., 2000).The bacteriocin activity was determined by the highest twofold dilution showing a clear inhibition zone on the MRS agar.

RESULTS AND DISCUSSION

Isolation and identification of Lactic Acid Bacteria.

A total of twenty isolates of lactic acid bacteria were obtained from ten samples of dairy products (curd, cheese and butter) on MRS agar plates in aerobic condition at 37^0C.All isolates were considered lactic acid bacteria based on their gram reaction and absence of catalase activity. All isolates were gram +ve, non spore forming, non motile rods. Isolates showed production of acid and gas from glucose while only acid from lactose and maltose. The isolates were non-spore formers. They are oxidase negative. They showed Hugh-Leifsons test positive for fermentation of glucose. They showed Indole & VP test – ve while MR tests + ve.

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Growth inhibition activity of LAB isolates against test organisms.

Thirteen isolates of lactic acid bacteria were able to inhibit growth of test cultures. The diameters of zones of growth inhibition (including diameter of cup) ranges from 7 to 22 mm, thus it was implied that the inhibitory substances were produced during growth of lactic acid bacteria. The LAB – VI isolate showing largest zone of growth inhibition was selected for study of their probiotic properties.

Probiotic Properties

LAB – VI isolate was susceptible to ciprofloxacin, gentamycin, ofloxacin, streptomycin and ampicillin. As the LAB is susceptible to all the antibiotics, there is no question of transfer of resistance to pathogenic organisms.

LAB – VI isolate can tolerate acid stress (pH: 3.0) up to 3hrs. All test strains survived on incubation period of 3 hrs. at pH 3.0 with decrease in survival percentage as exposure time progresses.

Tolerance to bile salts is considered to be prerequisite for colonization & metabolic activity of bacteria in the small intestine of host. Therefore, when evaluating the potential of using lactic acid bacteria as effective probiotic it is generally considered necessary to evaluate their ability to resist the effect of bile salts. In this study, the bile tolerance of LAB – VI isolate was investigated. LAB-VI isolate survived at 0.3% W/V bile salt concentration during 4 hrs.

Optimization of growth conditions for maximum growth of LAB – VI isolate

Maximum growth of LAB – VI isolate in MRS broth was observed at pH: 5(Figure: 1), inoculum size 4% v/v (Figure: 2) & incubation period 48 hrs (Figure: 3).

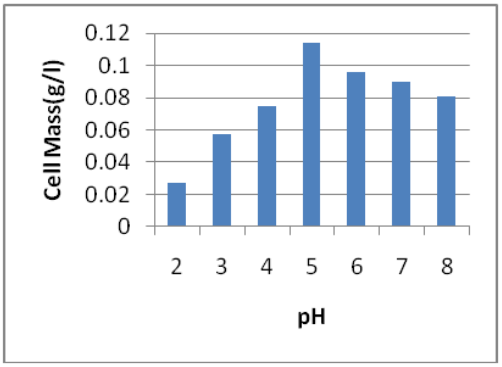

Figure: 1- Effect of pH on growth

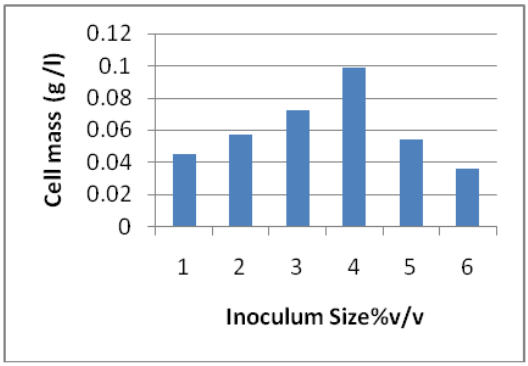

Figure: 2-Effect of Inoculum Size on growth

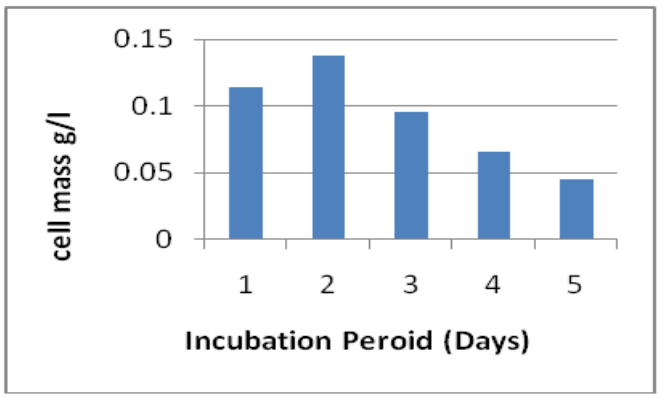

Figure: 3- Effect of Incubation Period on growth

Effect of coconut extract as a raw material substitute in MRS broth on growth of LAB – VI isolate under optimized conditions.

LAB – VI isolate was grown in a coconut extract media of two different compositions.

The growth of LAB- VI isolate & various products formation is reported in Table: 3

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Table 3: Summary of effect of composition of coconut extract medium (Type I and II) on growth & production of antimicrobial substances

Type of coconut extract medium	Incubation time (hrs)	Cell mass (gms/lit)	Quantity of lactic acid (mgs)	Amount of H_2O_2 (mgs)	Quantity of glucose (gms/lit)	PH Change
Type I	0 hrs	0.003	15.3	18.2	0.036	5.0
	4 hrs	0.009	17.1	21.4	0.036	4.8
	8 hrs	0.015	18.9	22.5	0.024	4.2
	24 hrs	0.024	21.6	23.5	0.009	3.9
Type II	0 hrs	0.009	16.2	20.3	0.042	5.0
	4 hrs	0.021	18.0	21.4	0.039	4.9
	8 hrs	0.024	19.8	24.6	0.027	4.7
	24 hrs	0.03	22.5	24.6	0.012	4.4

Results shows pH drop with increment of lactic acid content. The increase in the production of lactic acid with time has been attributed to lowered pH. The increase in lactic acid content, drop in pH and the decrease concentration of glucose with time are due to utilization of substrate (Xanthopoulos, et al., 1997).The LAB-VI isolate produced antimicrobial compounds.

Coconut is the source of important physiological functional components comprising carbohydrate, sugars, dietary fiber, fat, protein, amino acids & minerals (Creswell, et al., 1991). Higher bacterial growth was recorded in Type II than in Type I coconut extract medium may be as a result of enrichment of Type II coconut extract medium with peptone, Beef extract, yeast extract and glucose. Less ability of Type I coconut extract medium to induce biomass synthesis may be as a result of the fact that coconut extract medium does not contain enough nitrogen source needed by lactic acid bacteria. Lactic acid bacteria are fastidious microorganisms that require complex nutritional substances for growth and biosynthesis (Tanuwat, 1991). .Type II medium has less potential when compared for biomass production with MRS broth. This is because coconut extract medium has complex nitrogen source and selected isolate is unable to metabolize the complex nitrogen source present (Adebayo- Tayo, et al., 2011). Therefore there is a need for partial hydrolysis of the native protein and complex carbohydrate in the substrate for growth of LAB-VI isolate. This necessitates further research work.

Bacteriocin Activity

The bacteriocin activity of two types of Bacteriocin (Bacteriocin from fermented Type I & Type II coconut extract media) were 1:6 & 1:8 respectively (Table: 4).

Table 4: Bacteriocin Activity

S. No.	Bacterial isolate	Type of coconut extract media	Different dilutions of Bacteriocin (Test organism *Klebsiella aerogens*)					
			Pure	1:2	1:4	1:6	1:8	1:16
1	LAB – VI	Type I	16 mm	11 mm	10 mm	8mm	-------	-------
2	LAB – VI	Type II	20 mm	17 mm	11 mm	9mm	8 mm	-------

CONCLUSION

In conclusion, the formulated medium with a cheap and natural renewable source can be used as a substitute substrate for the biomass production of LAB VI isolate. The investigation focuses on the use of coconut extract as a partial substitute for complex nitrogen sources, namely peptone, yeast extract, and Beef extract for the production of biomass.

It is evident that coconut extract can be used to decrease the initial concentrations of the nitrogen source in the modified medium by about 50%.Coconut extract is cheap, therefore, cost of production of the biomass of LAB – VI isolate obtained using coconut extract medium was less as compared MRS medium, therefore Type II coconut extract medium can be used as substitute of MRS medium. The potential of the LAB VI isolate to inhibit the food pathogens, *Klebsiella aerogens, S. aureus, pseudomonas aeruginosa, salmonella spp. & Ps. mirabilis* make it of crucial interest especially in processed foods where there is risk of food pathogens.

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