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Original Research Article

Comparative study of the effect of various parameters on growth and antimicrobial activity of probiotics

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A B S T R A C T

Keywords

Lactobacillus casei, Lactobacillus fermentum, Streptococcus thermophillus

The *Lactobacilli* shows pronounced antibacterial activity between early logarithmic and early stationary phase. The maximum activity in composed medium was achieved at initial pH of 6, incubation temperature 37°C, and incubation period of 24h under static condition. Supplementation or replacement of nutrients demonstrated that larger quantities of growth could be produced. The compound produced by *Lactobacilli* was fully inactivated by some of the proteolytic enzymes, which confirms their proteinaceous nature. This study aimed to modify de Man Rogosa Sharpe culture medium (termed MRS) for selective cultivation of probiotics strain.

Introduction

Probiotics are an important part of the complex world of foods that are good for health. These bacteria have belong to the natural flora in order to be able to resist acid and bile, to survive during intestinal transit, to adhere to the intestinal mucosa and to produce antimicrobial substances in order to retain the characteristics that contribute to their beneficial health effects (Fuller, 1989). Probiotic organisms are thought to act through a variety of mechanisms which includes (a) Compete with potential pathogens for nutrients and growth factors or enterocyte adhesion sites or substract and involved in modifying the gut pH. (b) Degradation of toxins, production of antimicrobial substances, and antioxidants (Caglar *et al.*, 2006). The lactic acid bacteria need to withstand varying environmental conditions including differences in temperature, pH and salinity, depending on the specific application. Glucose is frequently used as a carbon source in lactic acid fermentation. For instance, it had been used to promote lactic acid fermentation of shrimp wastes (Rao *et al*. 2000). For application of these strains in processes where high salt concentrations are required or are naturally present, their salt tolerance should be studied. Combined effect of low initial pH and salt addition may present additional advantages.

Materials and Methods

Microorganisms: Bacterial pure cultures *L. casei, L. fermentum, L .rahamnosus* and *S. thermophillus* were obtained from culture collection of National Dairy Research Institute (NDRI), Karnal, and the stock cultures were maintained by periodic subculture and Lactobacillus Man Ragosa& Sharp (MRS) Agar Medium (4^0C).

Culture media: The cultures *L. casei, L. fermentum, L. rahamnosus* and *S. thermophillus* were inoculated on MRS agar and incubated at 37^0C for 24 hr until extensive growth of colonies were seen.

Preparation of inoculum: Growth of organism appeared on Lactobacillus MRS Agar Medium after 24–48hrs of incubation, and the inoculum was transferred to 5% peptone water with inoculation loop. The inoculum strength was checked at 540nm and set at OD 0.3 to be used as inoculums when OD is 1.0 the dry weight is 0.28 (Rosemarei *et al.*, 1989).

Growth analysis of probiotics using different parameters

Comparative growth on different selective media

Different media selected were followed as:

1. MRS broth
2. Tomato juice broth (TJB)
3. Tryptone glucose yeast extract (TGYE)

With all standard and optimal conditions, various selective media were examined as optimal media for growth of probiotic bacteria. 1ml of inoculums, with OD 0.3 at 540 nm was added to various media (9ml) and incubated at 37°C. Variable media of same concentration are used, results were recorded for 4 days with an interval of 24 hrs compared with reference to control.

Comparative growth on variable carbon sources

Different organic carbon sources selected were as:

1. Glucose
2. Fructose
3. Maltose

With all standard and optimal conditions, an additional source of carbon was used to examine as nutrient sources for given cultures of bacteria. 20gm/L concentration of various sources was selected. 1ml of inoculum, with OD 0.3 at 540 nm was added to MRS media (9ml) and incubated at 37°C. Variable sources of same concentration were used, results were recorded for 4 days with an interval of 24 hrs compared with reference to control.

Comparative growth on various nitrogen sources:

An additional source of nitrogen (organic) was used to examine as nutrient for growth of given cultures of bacteria. Variable nitrogen sources (peptone 10 g/L, Yeast extract 5g/L, Casein 5g/L) of same concentration were used and 1ml of inoculum, with OD 0.3 at 540 nm was added to MRS media (9ml) and incubated at 37°C. Results were recorded for 4 days incubation with an interval of 24 hrs compared with reference to control.

Comparative growth on various phosphorous sources:

Various source of phosphorous (KH_2PO_4, K_2HPO_4) inorganic, was used to examine as nutrient for growth of given cultures of bacteria. 1ml of inoculum, with OD 0.3 at 540 nm was added to MRS media (9ml) and incubated at 37°C. Variable sources of phosphorous of same concentration were used and results were recorded for 4 days

incubation with an interval compared with reference to control.

Comparative growth on various pH:

An additional condition of variable temperature pH 5.5, pH 6.5 & pH 7.5 was used to examine as condition for growth of given cultures of bacteria. 1ml of inoculum, with OD 0.3 at 540 nm was added to MRS media (9ml) and incubated at 37°C. Variable temperature was used and results were recorded for 4 days incubation with an interval of 24 hrs compared with reference to control.

Comparative growth on various temperatures:

An additional condition of variable temperature 32°C, 37°C and 42°C was used to examine as condition for growth of given cultures of bacteria. 1ml of inoculum, with OD 0.3 at 540 nm was added to MRS media (9ml) and incubated at 37°C. Variable temperature was used and results were recorded for 4 days incubation with an interval of 24 hrs compared with reference to control.

Determination of antimicrobial activity of Probiotics – test pathogens by well diffusion method and over lay method

Well Diffusion Method: Two strains of pathogens were included in study (*E. coli*, *S. typhi*). Selective media were used to test the anti microbial activity against these pathogens. 0.1 ml dilution of each pathogen were tested by pour plate method, four holes was made by using sterile cork borer and then pure cultures were added and results were recorded for 3 days incubation with 24 hrs interval .

Overlay method: Two strains of pathogens were included in study (*E. coli*, *S. typhi*) selective media were used to test the anti microbial activity against these pathogens. 0.1 ml dilution of each pathogen were tested by pour plate method, incubated anaerobically for 4 days for growth, then an another layer of media having either pure culture and isolated culture was over laid on it, results were recorded after 4 days of anaerobic incubation

Results and Discussion

It was inferred that *L. casei* grew well on peptone as additional organic nitrogen sources and rest three strains grew well on casein that is in contrast to Nebra and Blanch (1999) who observed no effect of casein on *Bifidiobacterium* spp and there was little stimulation in case of yeast extract that is in accordance to Todorov and Dicks (2005) and they observed stimulation by tryptone and no stimulation by yeast extract.

Studies on inorganic phosphorous sources

(a) *L. casei*

Use of different inorganic phosphorous sources was examined and it was observed according to data given in Table 12(a). According to data growth in KH_2PO_4 is highest 64% in comparison to K_2HPO_4.

(b) *L. fermentum*

Use of different inorganic phosphorous sources was examined and it was observed according to data given in Table according to data growth in K_2HPO_4 is highest 66% in comparison to KH_2PO_4

(c) *L. rahmnosus*

Use of different inorganic phosphorous sources was examined and it was observed according to data given below in Table according to data growth in KH_2PO_4 is

highest 64% in comparison to K_2HPO_4.

(d) *S. thermophillus*

Use of different inorganic phosphorous sources was examined and it was observed according to data given below in Table 6.1(a). According to data growth in $K_2HPO_4$is highest 35% in comparison to KH_2PO_4

It was concluded that *L. casei* and *L. rahmnosus* gave high percentage increase in KH_2PO_4 as inorganic phosphorous sources and no stimulation by K_2HPO_4 that may be due to low concentration as observed by Todorov and Dicks (2005) and rest of strains gave high percentage increase in K_2HPO_4 as inorganic phosphorous sources. Mitchell (1954) showed that maximum inhibitory effect of probiotics by uptake of H_2PO_4 rather than HPO_4.

Studies using inorganic carbon sources

(a) *L. casei*

Use of different inorganic carbon sources was examined and it was observed according to data given in table. According to data, growth in $CaCO_3$ is highest 135% in comparison to ammonium carbonate and H_2CO_3.

(b) *L. fermentum*

Use of different inorganic carbon sources was examined and it was observed according to data given in table. According to data growth in ammonium carbonate is highest 135% in comparison to $CaCO_3$ and H_2CO_3.

(c) *L. rahmnosus*

Use of different inorganic carbon sources was examined and it was observed according to data given in table. According to data growth in Ammonium carbonate is highest 135% in comparison to $CaCO_3$ and H_2CO_3.

(d) *S. thermophillus*

Use of different inorganic carbon sources was examined and it was observed according to data given below in table. According to data growth in $CaCO_3$ is highest 82% in comparison to Ammonium carbonate and H_2CO_3.

The comparative growth on various pH

The data given below shows that optimum pH for *L. casei, L. rahmnosus, L. fermentum, S. thermophillus* shows that optimum pH is 6.5.

It has been inferred that optimum pH for growth of these cultures is pH 6.5 but probiotics are taken orally and it has to pass through gastrointestinal tract and has to with stand various conditions i.e. acidic. So probiotics can not only persist in different conditions but can also grow Rishi *et al* (2008).

The comparative growth on different temperatures

The data given below shows those optimum temperatures for *L. casei, L. rahmnosus, L. fermentum, S. thermophillus*is 37°C. It was concluded that optimum temperature for all four culture grow well at 37°C but *S. thermophillus* showed good growth at 42°C .

Determination of antimicrobial activity of Probiotics – Test pathogens by Well Diffusion Method and Overlay method.

Data presented in table given involves the use of agar well method with reference to sensitivity of test pathogens in presence of pathogens. Antimicrobial sensitivity

showing zone of inhibition against *E. coli*. Result of the agar overlay method showed that all of the probiotic strains were showing inhibiton against test microbial isolates. The spectrum of their antimicrobial activity varied.

Table.1(a) Studies using different growth media

Percentage increase in biomass	TGYE	MRS	TJB
Time Period (hrs)	Concentration of biomass (mg dry wt/ml)		
24	0.3696	0.237	0.580
48	0.0047	0.0865	0.926
72	0.358	0.4284	0.7112
96	0.629	0.794	0.688

Table.2(b) Studies using different growth media

Percentage increase in biomass	TGYE	MRS	TJB
Time Period (hrs)	Concentration of biomass (mg dry wt/ml)		
24	0.3696	0.414	0.823
48	0.0047	0.257	1.004
72	0.358	0.616	0.756
96	0.629	0.628	0.956

Table.3(c) Studies using different growth media

Percentage increase in biomass	TGYE	MRS	TJB
Time Period (hrs)	Concentration of biomass (mg dry wt/ml)		
24	0.338	0.336	0.368
48	0.003	0.017	0.951
72	0.358	0.428	0.062
96	0.629	0.435	0.639

Table.4(d) Studies using different growth media

Percentage increase in biomass	TGYE	MRS	TJB
TIME PERIOD (hrs)	Concentration of biomass(mg dry wt/ml)		
24	0.431	0.50	0.56
48	0. 780	0.219	1.02
72	0.411	0.632	0.719
96	0.670	0.641	0.815

Table.5(a) Studies using different organic carbon sources

Percentage increase in biomass	Glucose	Lactose	Maltose
Time Period (hrs)	Concentration of biomass(mg dry wt/ml)		
24	0.2452	0.264	0.258
48	0. 4816	0.3822	0.625
72	0.387	0.5118	0.561
96	0.389	0.4566	0.588

Table.6(b) Studies using different organic carbon sources

Percentage increase in biomass	Glucose	Lactose	Maltose
Time Period (hrs)	Concentration of biomass(mg dry wt/ml)		
24	0.2198	0.279	0.2447
48	0. 426	0.455	0.430
72	0.4211	0.451	0.458
96	0.408	0.4382	0. 459

Table.7(c) Studies using different organic carbon sources

Percentage increase in biomass	glucose	lactose	Maltose
Time Period (hrs)	Concentration of biomass(mg dry wt/ml)		
24	0.222	0.308	0.3612
48	0. 447	0.449	0.503
72	0.4886	0.460	0.530
96	0.495	0.507	0. 4919

Table.8(d) Studies using different organic carbon sources

Percentage increase in biomass	glucose	lactose	Maltose
Time Period (hrs)	Concentration of biomass(mg dry wt/ml)		
24	0.229	0.28	0.313
48	0435	0.497	0.456
72	0.447	0.479	0.4312
96	0.447	0.482	04244

Table.9(a) Studies using different organic nitrogen sources

Percentage increase in biomass	glucose	lactose	Maltose
Time Period (hrs)	Concentration of biomass(mg dry wt/ml)		
24	0.856	0.276	0.245
48	0.7896	0.337	0.482
72	0.5596	0.766	0.487
96	0.2904	0.656	0.386

Table.10(b) Studies using different organic nitrogen sources

Percentage increase in biomass	glucose	lactose	Maltose
Time Period (hrs)	Concentration of biomass(mg dry wt/ml)		
24	0.275	0.437	0.267
48	0.491	0.573	0.493
72	0.515	0.634	0.600
96	0.454	0.572	0.484

Table.11(c) Studies using different organic nitrogen sources

Percentage increase in biomass	glucose	lactose	Maltose
Time Period (hrs)	Concentration of biomass(mg dry wt/ml)		
24	0.535	0.525	0.279
48	0.501	0.342	0.399
72	0.530	0.684	0.576
96	0.490	0.654	0.538

Table.12(d) Studies using different organic nitrogen sources

Percentage increase in biomass	glucose	lactose	Maltose
Time Period (hrs)	Concentration of biomass(mg dry wt/ml)		
24	0.313	0.595	0.336
48	0.456	0.628	0.414
72	0.506	0.659	0.620
96	0.313	0.595	0.336

Table.13(a) Studies using different inorganic phosphorous sources

percentage increase in biomass	KH_2PO_4	K_2HPO_4
Time Period (hrs)	Concentration of biomass(mg dry wt/ml)	
24	0.279	0.560
48	0.382	0.625
72	0.5118	0.561
96	0.458	0.588

Table.14(b) Studies using different inorganic phosphorous sources

Percentage increase in biomass	KH_2PO_4	K_2HPO_4
Time Period (hrs)	Concentration of biomass(mg dry wt/ml)	
24	0.257	0.258
48	0.380	0.262
72	0.432	0. 498
96	0. 377	0.428

Table.15(c) Studies using different inorganic phosphorous sources

Percentage increase in biomass	KH_2PO_4	K_2HPO_4
Time Period (hrs)	Concentration of biomass(mg dry wt/ml)	
24	0. 239	0.220
48	0.4186	0.289
72	0.456	0. 3514
96	0. 397	0.355

Table.16(d) Studies using different inorganic phosphorous sources

Percentage increase in biomass	KH_2PO_4	K_2HPO_4
Time Period (hrs)	Concentration of biomass(mg dry wt/ml)	
24	0. 279	0.251
48	0.426	0.432
72	0.478	0. 596
96	0. 366	0.7411

Table.17(a) Studies using different inorganic carbon sources

Percentage increase in biomass	$CaCO_3$	Ammonium carbonate	H_2CO_3
Time Period (hrs)	Concentration of biomass(mg dry wt/ml)		
24	0.267	0.238	0.272
48	0.460	0.322	0.336
72	0.848	0.440	0.738
96	0.563	0.375	0.096

Table.18(b) Studies using different inorganic carbon sources

Percentage increase in biomass	$CaCO_3$	Ammonium carbonate	H_2CO_3
Time Period (hrs)	Concentration of biomass(mg dry wt/ml)		
24	0.245	0.275	0.247
48	0.2979	0.181	0.327
72	0.769	0.343	0.401
96	0.362	0.397	0.264

Table.19(c) Studies using different inorganic carbon sources

Percentage increase in biomass	$CaCO_3$	Ammonium carbonate	H_2CO_3
Time Period (hrs)	Concentration of biomass(mg dry wt/ml)		
24	0.250	0.257	0.247
48	0.2979	0.181	0.327
72	0.769	0.343	0.401
96	0.362	0.397	0.246

Table.20(d) Studies using different inorganic carbon sources

Percentage increase in biomass	$CaCO_3$	Ammonium carbonate	H_2CO_3
Time Period (hrs)	Concentration of biomass(mg dry wt/ml)		
24	0.213	0.274	0.217
48	0.387	0.306	0.2614
72	0.589	0.496	0.362
96	0.387	0.371	0.258

Table.21(a) Studies using different pH

pH	pH5.5	pH6.5	pH7.5
TIME PERIOD (hrs)	Concentration of biomass(mg dry wt/ml)		
24	0.470	0.5516	0.434
48	0.966	0.99	0.98
72	1.038	1.07	1.753
96	0.190	0.229	0.221

Table.22(b) Studies using different pH

Percentage increase in biomass	pH5.5	pH6.5	pH7.5
Time Period (hrs)	Concentration of biomass(mg dry wt/ml)		
24	0.644	0.63	0.523
48	0.988	1.008	1.01
72	1.055	1.07	1.1
96	0.794	0.263	0.2436

Table.23(c) Studies using different pH

pH	pH 5.5	pH6.5	pH7.5
TIME PERIOD (hrs)	Concentration of biomass(mg dry wt/ml)		
24	0.63	0.627	0.646
48	0.99	1.01	1.01
72	1.09	1.1	1.11
96	0.19	0.243	0.240

Table.24(d) Studies using different pH

Percentage increase in biomass	pH5.5	pH6.5	pH7.5
TIME PERIOD (hrs)	Concentration of biomass(mg dry wt/ml)		
24	0.512	0.512	0.56
48	0.9716	0.9716	0.912
72	1.09	1.09	1.07
96	0.222	0.222	0.238

Table.25(a) Studies using different temperatures

Temperature	32°C	37°C	42°C
TIME PERIOD (hrs)	Concentration of biomass(mg dry wt/ml)		
24	0.442	0.613	0.476
48	0.926	0.996	0.994
72	1.10	1.052	1.10
96	0.227	0.213	0.225

Table26(b) Studies using different temperature

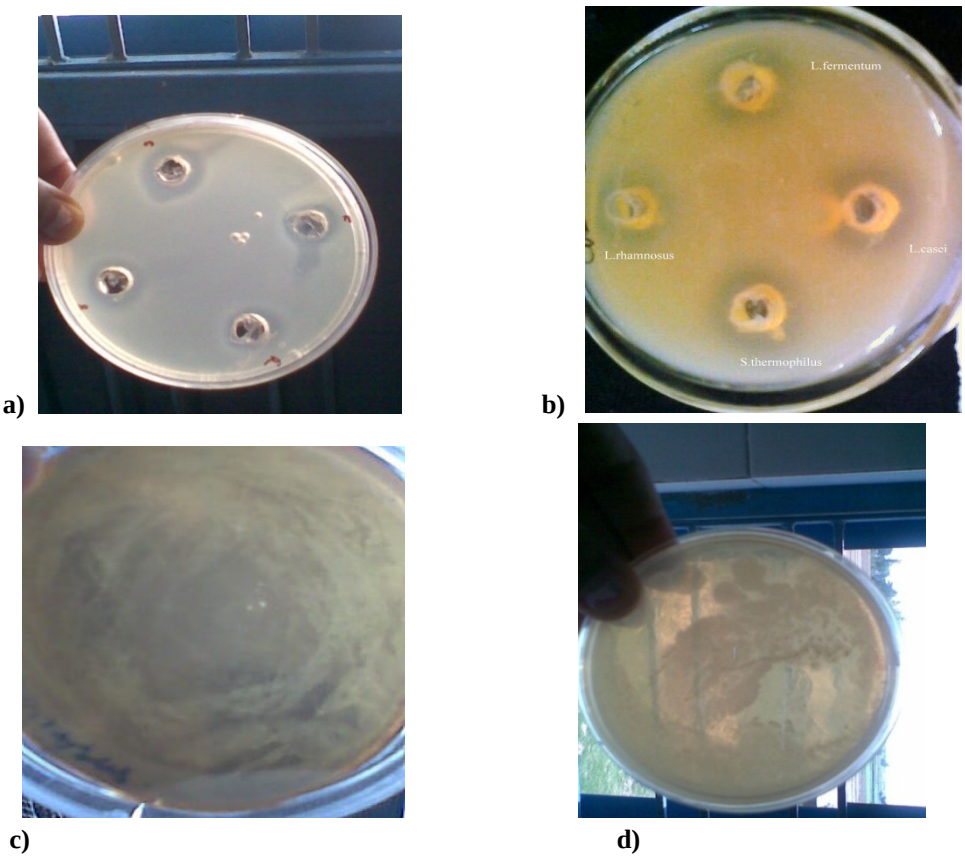
Temperature	32°C	37°C	42°C
TIME PERIOD (hrs)	Concentration of biomass(mg dry wt/ml)		
24	0.554	0.618	0.554
48	1.005	1.03	1.02
72	1.12	1.153	1.3
96	0.1988	0.170	0.21

Table.27(c) Studies using different temperature

Temperature	32°C	37°C	42°C
TIME PERIOD (hrs)	Concentration of biomass(mg dry wt/ml)		
24	0.621	0.537	0.585
48	1.02	1.036	1.008
72	1.14	1.122	1.052
96	0.24	0.2184	0.224

Table.28(d) Studies using different temperature

Temperature	32°C	37°C	42°C
Time Period (hrs)	Concentration of biomass(mg dry wt/ml)		
24	0.504	0.453	0.1792
48	0.952	1.03	0.9408
72	1.08	1.11	1.117
96	0.1876	0.204	0.229



Figures.16 a, b, c & d Results of Agar Well method (Figures a&b) and overlay method (Figures c&d)

Table.29 Zone of inhibition against test pathogens

Test organism	ZONE (Diameter) OF INHIBITION			
	L.casei	***L.fermentum***	***L.rahamnosus***	***S.thermophillus***
E.coli	17mm	19mm	15mm	12mm
S.typhi	15mm	10mm	12mm	09mm

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