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Full Length Research Paper

Influence of growth conditions and nutritional requirements on the production of hydrogen peroxide by lactic acid bacteria

Abimbola Enitan[1]*, Josiah Adeyemo[1] and S. T. Ogunbanwo[2]

[1]Durban University of Technology, P. O. Box 1334, Durban, 4000, South Africa.
[2]Department of Botany and Microbiology, University of Ibadan, Oyo State, Nigeria.

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Lactic acid bacteria (LAB) were isolated from raw and fermented milk samples. They were evaluated for hydrogen peroxide (H_2O_2) production. Sixty–three strains of LAB belonging to the genera *Lactobacillus, Leuconostoc, Lactococcus* and *Streptococcus* were isolated and all the isolates produce H_2O_2 with *Lactococcus lactis* having the highest yield of 0.4279 mg/L in de Mann Rogosa Sharpe (MRS) broth. Among the high–level H_2O_2-producing strains *are Lactobacillus bulgaricus, Lactobacillus casei, L. lactis* and *Streptococcus thermophiles.* They were subsequently selected and their culture supernatants were evaluated at different temperatures, pH values and using different carbon and nitrogen sources. The highest quantity of H_2O_2 (0.6517 mg/L) was produced at 37°C by *S. thermophilus*, while *L. casei* produced the lowest quantity of 0.1132 mg/L at 40°C . *S. thermophilus* produced the highest quantity of 0.5912 mg/L at pH 5.5, while the lowest quantity of 0.1042 mg/L was produced at pH 7. *L. bulgaricus* produced the highest concentration of0.6512 mg/L when galactose was used as carbon source and lowest quantity of 0.0210 mg/L was produced by *L. lactis* in basal medium containing sorbitol as carbon source. *L. casei* produced both the highest and lowest quantities of 0.1895 and 0.0207 mg/L of H_2O_2 in medium supplemented with yeast extract and ammonium sulphate as nitrogen sources respectively. The antimicrobial activity of the culture supernatants against *Escherichia coli* K12, *Staphylococcus aureus* and *Candida albicans* using agar well diffusion assay was evaluated. Cell-free supernatant by *L. bulgaricus* had the highest inhibitory activity against *E. coli* K12 with 21 mm zone of inhibition and against *S. aureus* with 15 mm zone of inhibition but showed no antagonistic activity against *C. albicans*. The study revealed that lactic acid bacteria isolated from raw and fermented milk in South-West Nigeria are capable of producing hydrogen peroxide which has antagonistic effect on pathogenic organisms, thus, may be promising sources of preservative that may in future be applied to food.

Key words: Hydrogen peroxide, growth conditions, nutrient utilization, antagonistic activity, lactic acid bacteria (LAB).

INTRODUCTION

Lactic acid bacteria (LAB) are found in many nutrient rich environments and occur naturally in various food products such as diary, meat products and vegetables (Carr et al., 2002; Mallesha et al., 2010; Jones et al., 2008). LAB, especially *Streptococcus, Leuconostoc Lactococcus and Lactobacillus* have long been used as starters in food fermentation where they exert technologically important functions such as acidification, aroma formation, modification of rheological properties and contribution to the taste and texture of fermented foods (Axelsson, 2004; Abdullah and Osman, 2010). The antimicrobial compounds produced by LAB include organic acids, diacetyl, hydrogen peroxide or bacteriocins (Sanni

*Corresponding author. E-mail: enitanabimbola@gmail.com. Tel: +27313732597. Fax: +27313732778.

et al., 1995; Ogunbanwo et al., 2004). Production of these compounds by certain LAB strains contributed to their inhibitory activity against other microorganisms including food borne pathogens, food preservation and prevention of certain diseases (Ito et al., 2003).

The synthesis of H_2O_2 by LAB in the environment is believed to occur as a result of the action of flavoprotein oxidases or nicotinamide adenine dinucleotide (NADH) peroxidase. It may also be as a precursor for the production of bactericidal free _ radicals such as superoxide (O^{-}_2) and hydroxyl (OH) radicals which can damage DNA (mmor et al., 2006). The production of H_2O_2 by LAB strains can be detected in de Mann Rogosa Sharpe Medium (MRS). The medium compositions greatly influence the ability of LAB to produce H_2O_2. Berthier (1993) found that the lower the glucose concentration, the better the H_2O_2 production irrespective of the medium used.

The production of H_2O_2 by LAB isolated from traditional Nigerian fermented foods and beverages and its antimicrobial effect have been reported by many researchers. Dike and Sanni (2010) did some work on the influence of starter culture of LAB to control spoilage of agidi, a cereal based African fermented food. It was reported that *Aspergillus flavus* and *Aspergillus niger* were inhibited by *Lactobacillus casei* isolated from agidi. The production of hydrogen peroxide by LAB isolated from some traditional fermented foods and its antimicrobial activity on *Pseudomonas aeruginosa* were also been examined by Adesokan et al. (2010).

Consumption rate of raw milk, cheese, yogurt and other fermented products is high in Nigeria thus, if they are to be produced on a large scale, some tasks have to be undertaken because, the use of synthetic chemicals as food preservatives is becoming a serious concern to the consumers. Therefore, it is logical to characterize hydrogen peroxide producing-LAB that are effective over a wide range of environmental conditions in other to improve the safety of fermented and processed food products.

In the light of the above, this present investigation was aimed at examining the influence of a broad range of growth conditions and nutritional requirements on the growth and production of H_2O_2 by fermentation using LAB as test isolates. To evaluate the inhibitory effect of hydrogen peroxide produced by LAB on the pathogenic strains of *Candida albicans, Escherichia coli* and *Staphylococcus aureus in vitro*.

MATERIALS AND METHODS

Test (indicator) strains

C. albicans (clinical isolate), *E. coli* and *S. aureus* were obtained from the Medical Microbiology Unit, University College Hospital, Ibadan, Nigeria and Environmental Laboratory, Department of Botany and Microbiology, University of Ibadan, Nigeria and were used as test microorganisms.

Collection of samples

Fresh sheep and goat milk samples were collected from Bodija Market Ranch, Ibadan while yoghurt and cheese samples were obtained from local producers at the local market at Ojoo, Ibadan, South-West Nigeria. Samples were conveyed to the Department of Botany and Microbiology University of Ibadan Laboratory in sterile containers for immediate microbiological analysis.

Isolation and identification of lactic acid bacteria

Organisms were isolated from the samples collected using pour plate method. Preliminary identification of the isolates `were done according to Bergey's Manual of Systematic Bacteriology (Sneath, 1986) based on cell and colony morphology, Gram staining, catalase test, spores staining, growth at 15°C and 45°C, motility test and other biochemical tests such as indole production, Voges–Proskauer test, oxidase test, methyl red test, production of ammonia from arginine, growth in 4% NaCl broth and carbohydrate fermentation pattern. API 20 Strep test kits (Biomerioux, France) was used for further identification of the presumptive isolates to species level. The isolated LAB were placed in MRS broth with 20% sterile glycerol and stored at -80°C before further screening.

Hydrogen peroxide production

All isolated LAB from the stock cultures were inoculated onto MRS agar and then subcultured into MRS broth (pH 7.0). After 72 h incubation at 30°C, the cultures were centrifuged at 10,000 rpm for 20 min at 4°C. 5 mg/ml Protenase K was added to cell free extract solution and pH was adjusted to 7.0 by means of 0.1N NaOH to exclude the antimicrobial effect of bacteriocins and organic acid respectively, followed by filtration of the supernatant through a 0.2 μm pore size cellulose acetate filter. Known volume of the supernatant was used for the titration at 12 h interval to determine the quantity of hydrogen peroxide produced. 25 ml of supernant of broth cultures of the test organisms was measured into a 100 ml flask. To this was added 25 ml of dilute H_2SO_4. This was then titrated with 0.1 N potassium permangenate($NKMn0_4$). Each milliliter of 0.1 N $NKMn0_4$ is equivatent to 1.701/mg of H_2O_2. A decolourization of the sample was regarded as the the end point. The volume of H_2O_2 produced was then calculated (AOAC, 1990). All experiments were performed in duplicate and the concentration of H_2O_2 produced by isolates was calculated thus:

$$H_2O_2 \text{ concentration} = \frac{Ml\ KMnO_4 \times NKMnO_4 M.E \times 100}{Ml\ H_2SO_4 \times \text{volume of sample}}$$

Ml $KMnO_4$ = volume of $KMnO_4$ used, N $KMnO_4$ = concentration of $KMnO_4$ used, M.E = equivalence factor, ml H_2SO_4 = volume of H_2SO_4 added to the sample.

Effect of temperature on H_2O_2 production

Approximately 10^8 cfu of lactic acid bacteria was inoculated into 10 ml of sterile MRS broth. The tubes were incubated at 30, 37 and 40°C. Samples were collected at 12 h interval and H_2O_2 produced were quantified as stated above. The growth was determined at A_{600} using spectophotometer.

Effect of initial pH on H_2O_2 production

The ability of lactic acid bacteria to produce H_2O_2 at different pH

was tested by culturing them in sterilized MRS broth with pH adjusted to pH 4, 5.5, 7 and 9 using hydrochloric acid or NaOH and inoculated at 37°C for 72 h. Samples were collected at 12 h interval and H_2O_2 produced was quantified as stated above (AOAC, 1990) and growth was determined at A_{600} using spectophotometer.

Influence of carbon sources on the production of H_2O_2

To test the influence of carbon sources the test organisms were grown in modified MRS broth containing different carbon sources. The basal medium contained 0.2 g $MgSO_4.7H_2O$, 0.05 g $MnSO_4$. $4H_20$, 5 g sodium acetate, 1.5 g of K_2HP0_4, 1.5 g of KH_2P04, 10 g peptone, 5 g yeast extract, 1 ml of Tween 80 per litre of distilled water and various amount of carbon such as galactose, lactose, starch and sorbitol at 2% concentration (w/v) were added. They were inoculated with 0.1 ml of overnight culture of the test organisms and incubated at 37°C with initial pH of 5.5. Samples were collected at 12 h interval and H_20_2 produced was quantified as stated above while the growth was determined at A_{600} using spectophotometer.

Influence of nitrogen sources on the production of H_2O_2

To determine the influence of different nitrogen sources, the test organisms were grown in basal medium containing 0.2 g $MgS0_4$. $7H_2$ 0, 0.05 g $MnS0_4$. $4H_2$ 0, 5 g sodium acetate, 1.5 g KH_2P0_4, 6% w/v glucose and 1.0 ml vitamin solution containning (per 100 ml 20% ethanol) 0.2 g vitamins B_6, 0.1 g riboflavin and 0.1 g folic acid per litre of distilled water and various amount of nitrogen – sources such as yeast extract, casein , urea, and $(NH_4)_2S0_4$ at 2% $^{w}/_{v}$ nitrogen concentration were added. They were inoculated with 0.1 ml of overnight culture of the test organisms and incubated at 37°C with initial pH of 5.5. H_2 0_2 was quantified at every 12 hours and the growth was determined at A_{600} using spectrophotometer.

Antagonistic activity of H_2O_2 produced by the test isolates

A well diffusion assay method was employed to test the ability of the lactic acid bacteria to inhibit pathogenic organisms using the cell-free supernatant. 18 h old culture of indicator organisms was used to inoculate nutrient agar plate. 6 mm diameter holes were created in the inoculated nutrient agar plate using a cork borer. Hydrogen peroxide produced by the LAB was dispensed into each of the holes. The plates were incubated aerobically at 30°C overnight. The diameter of the inhibition zones were taken to be proportion to the logarithms of the H_2O_2 concentration.

RESULTS

Sixty-three species of LAB were randomly isolated from the various samples of raw and fermented milk. They were characterized as nine species belonging to four genera and are identified as *Lactobacillus plantarum, Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus brevis*, *Leuconostoc mesenteroides, Lactococcus lactis* and *Streptococcus thermophilus* based on the cultural, morphological, biochemical tests and API 20 Strep kits test. The percentage occurrence of LAB isolates are shown in Table 1. *L. plantarum* had the highest occurrence of 24% from fermented milk while *L. bulgaricus* showed least occurrence of 2%. *L. mesenteroides* had 6% occurrence in cheese, while *L. lactis* and *S. thermophilus* in sheep milk had 9% occurrence each.

The LAB species were screened for the quantitative production of hydrogen peroxide using normal MRS broth and modified MRS broth. In the normal MRS broth, it was observed that *L. lactis* produced the highest quantity of H_2O_2 (0.4279 mg/L) at 48 h compared to all other LAB species used in this work, while *L. plantarum* had the lowest yield (0.1344 mg/L) at 72 h of fermentation (Figure 1).

The influence of incubation temperature on the production of H_2O_2 revealed that the highest quantity of H_2O_2 was produced at 37°C by all the test isolates with *S. thermophilus* recording the highest yield of 0.6517 mg/L with growth of 1.92 O. D at 37 °C in 48 h, while *L. casei* produced the lowest (0.1132 mg/L) at 40°C in 12 h incubation time (Table 2).

The optimum initial pH for production of H_2O_2 as shown in Table 3 was at pH 5.5. *S. thermophilus* produced the highest quantity of hydrogen peroxide (0.5912 mg/L) with growth of 1.92 O. D at pH 5.5 in 48 h of fermentation, while the lowest quantity of H_2O_2 (0.1042 mg/L) was produced by the same organism with growth of 0.39 O.D at pH 7 in 12 h incubation period.

As fermentation progressed, there was increase in quantity of H_2O_2 produced between 12 and 48 h of incubation, after which decline set in. It was observed that accumulation of H_2O_2 production caused the culture to enter into stationary phase and growth remained constant for several hours. The influence of different carbon sources on the production of H_2O_2 showed that all the organisms produced the highest quantity of H_2O_2 when galactose was used as a carbon source. H_2O_2 concentration was higher (0.6512 mg/L) when *L. bulgaricus* was cultured at 48 h of incubation in the basal medium containing galactose as carbon source with growth of 1.86 O.D; while *L. lactis* produced the lowest quantity of H_2O_2 (0.0210 mg/L) with growth of 0.77 O.D at 12 h of incubation in basal medium containing sorbitol as carbon source (Table 4).

Yeast extract greatly influenced the quantity of H_2O_2 produced by the test isolates, while other nitrogen sources had minimal effect. *L. casei* produced 0.1895 mg/L of H_2O_2 as its highest quantity with growth of 1.89 O.D at 48 h of fermentation when yeast extract was used as nitrogen source, while the lowest yield (0.0207 mg/L) with growth of 0.29 O.D was produced by the same organism in medium supplemented with ammonium sulphate as nitrogen source (Table 5).

Inhibitory activity of H_2O_2 produced by the test isolates against *E. coli, C. albicans* and *S. aureus* was investigated. H_2O_2 produced by the test isolates had antagonistic effect against *S. aureus* and *E. coli* K12 and showed no inhibitory activity against *C. albicans*. H_2O_2 – fluid produced by *L. bulgaricus* had the highest inhibitory activity against *E. coli* K12 with 21 mm zone of inhibition

Table 1. Percentage occurrence of LAB species isolated from raw and fermented milk.

Isolate	Sample	Number of isolates (n)	% of isolates
L. plantarum	Yoghurt	15	24
	Goat milk	2	3
	Cheese	2	3
L. casei	Yoghurt	7	11
L. bulgaricus	Yoghurt	1	2
L. fermentum	Goat milk	4	6
	Cheese	3	5
L. lactis	Goat milk	1	2
	Cheese	2	3
L. brevis	Goat milk	1	2
L. Mesenteriodes	Goat milk	5	8
	Cheese	4	6
L. Lactic	Cheese	1	2
	sheep milk	6	9
S. thermophiles	Goat milk	1	2
	Cheese	2	3
	sheep milk	6	9
Total		63	100%

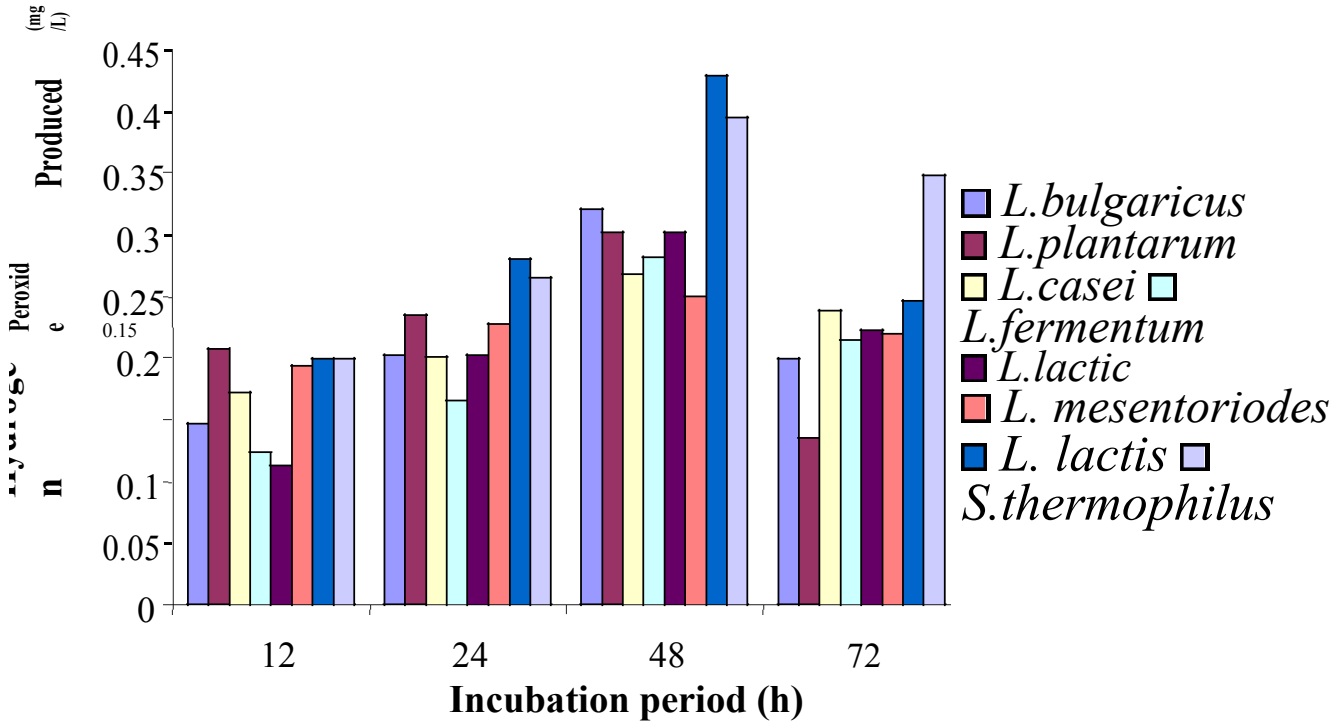

Figure 1. Quantity of hydrogen peroxide produced in MRS broth by the test isolates.

Table 2. Effect of temperature on the growth and H_2O_2 production by the test isolates

Temperature (°C)		Incubation period (h)							
		12		24		48		72	
		H_2O_2 produced		H_2O_2 produced		H_2O_2 produced		H_2O_2 produced	
	Test isolates	(mg/L)	Growth (O.D)	(mg/L)	Growth (O.D)	(mg/L)	Growth (O.D)	(mg/L)	Growth (O.D)
30	*L. bulgaricus*	0.1797	1.78	0.2675	1.86	0.2933	1.91	0.1976	1.9
	L. casei	0.1332	1.2	0.1848	1.22	0.3234	1.91	0.1419	1.9
	L. Lactis	0.2268	1.38	0.2814	1.67	0.4335	1.91	0.3865	1.9
	S. thermophilus	0.2814	1.89	0.3682	1.95	0.4826	1.92	0.2713	1.91
37	*L. bulgaricus*	0.2349	1.84	0.2864	1.89	0.3279	1.93	0.2108	1.91
	L. casei	0.1824	1.25	0.2871	1.79	0.3805	1.9	0.3409	1.9
	L. Lactis	0.2408	1.79	0.3516	1.91	0.5529	1.91	0.4132	1.92
	S. thermophilus	0.3566	1.88	0.384	1.92	0.6517	1.92	0.6322	1.92
40	*L. bulgaricus*	0.1475	0.66	0.2342	1.81	0.1951	1.9	0.1502	1.89
	L. casei	0.1132	1.29	0.1353	1.69	0.2417	1.89	0.1241	1.84
	L. lactis	0.1421	1.88	0.2675	1.91	0.4024	1.89	0.3404	1.84
	S. thermophilus	0.2164	1.88	0.3608	1.9	0.4489	1.9	0.2446	1.88

and against *S. aureus* with 15 mm zone of inhibition (Table 6).

DISCUSSION

Among the antimicrobial compounds produced by Lactic acid bacteria is hydrogen peroxide and it was closely examined in this study. The entire test LAB produced H_2O_2 with peak production at 48 h of incubation period followed by a decline in production at the end of 72 h fermentation with highest quantity produced by *L. lactis* and lowest by *L. plantarum.* Optimum temperature for the production of H_2O_2 by the test isolates was at 37°C by *S. thermophilus*, while *L. casei* produced the lowest quantity of H_2O_2 at 40°C. This trend indicates that increase in temperature up to an optimum level positively favors its production. Adesokan et al. (2010) reported the production of little H_2O_2 in 24 h and then larger amount of H_2O_2 with an increase in incubation time at 30°C.

Control of growth medium pH can also influence the H_2O_2 formation and stability. The best pH for the optimal production of hydrogen peroxide by the test isolates was at pH 5.5. This is in accordance with the reports of several workers such as (Barnard et al., 1999; Leroy and Vuyst, 1999; Marty-Teysset et al., 2000; Rebecca et al., 2008). Medium with an initial pH of 5.5 to 6.0 enhanced better LAB cell growth and product formation (Bai et al., 2004; Akerberg et al., 1998; Fowoyo and Ogunbanwo, 2010). Herranz et al. (2001) reported that the use of pH-controlled fermentation can lead to increase in the production of primary metabolites and growth rates. This might have contributed to the higher hydrogen peroxide production observed at this pH. Similarly, (Leroy and Vuyst, 1999) reported that bacteriocin activity was very much influenced by changes in temperature and pH. Ito et al. (2003) indicated that the presence of carbohydrates source was necessary for the production of H_2O_2 by lactobacilli. Therefore, the formation of H_2O_2 by the test isolates was examined and the entire test isolates best utilized galactose in modified MRS broth as carbon source for production of hydrogen peroxide when

Table 3. Effect of pH on the growth and H_2O_2 production by the test isolates.

pH		Incubation period (h)							
		12		24		48		72	
		H_2O_2 produced		H_2O_2 produced		H_2O_2 produced		H_2O_2 produced	
	Test isolates	(mg/L)	Growth (O.D)	(mg/L)	Growth (O.D)	(mg/L)	Growth (O.D)	(mg/L)	Growth (O.D)
4	*L. bulgaricus*	0.1935	1.9	0.3027	1.92	0.3561	1.92	0.3031	1.91
	L. casei	0.1562	1.8	0.2812	1.88	0.3471	1.9	0.2797	1.9
	L. lactis	0.1846	1.89	0.2675	1.9	0.4877	1.92	0.4862	1.92
	S. thermophilus	0.2245	1.87	0.2685	1.89	0.3764	1.91	0.342	1.91
5.5	*L. bulgaricus*	0.2397	1.9	0.3675	1.91	0.4933	1.93	0.3464	1.91
	L. casei	0.2714	0.75	0.2871	1.88	0.3805	1.9	0.3009	1.9
	L. lactis	0.2488	1.79	0.3425	1.87	0.5324	1.94	0.5132	1.94
	S. thermophilus	0.3346	1.88	0.4362	1.9	0.5912	1.92	0.5542	1.92
7	*L. bulgaricus*	0.1482	1.92	0.2964	1.92	0.3879	1.9	0.2938	1.83
	L. casei	0.1334	0.43	0.1882	1.78	0.3261	1.89	0.2839	1.78
	L. lactis	0.1832	1.62	0.2368	1.74	0.3213	1.8	0.2914	1.76
	S. thermophilus	0.1042	0.39	0.2419	0.74	0.3669	1.84	0.3417	1.78
9	*L. bulgaricus*	0.1464	0.66	0.2732	0.85	0.2833	1.21	0.2632	1.21
	L. casei	0.1089	0.92	0.1447	1.1	0.3167	1.49	0.2041	1.32
	L Lactis	0.1341	0.78	0.2137	0.95	0.2489	1.29	0.2304	1.29
	S. thermophilus	0.1082	0.88	0.2136	1	0.2299	1.4	0.2046	1.38

Table 4. Influence of carbon sources on growth and hydrogen peroxide production by the test isolates.

Carbon sources		Incubation period (h)							
		12		24		48		72	
		H_2O_2 produced							
	Test isolates	(mg/L)	Growth (O.D)	(mg/L)	Growth (O.D)	(mg/L)	Growth (O.D)	(mg/L)	Growth (O.D)
Galactose	*L. bulgaricus*	0.1448	1.24	0.2418	1.4	0.6512	1.86	0.2418	1.77
	L. casei	0.1532	1.09	0.1709	1.75	0.4253	1.85	0.1418	1.83
	L. lactis	0.1389	1.38	0.1417	1.46	0.3403	1.66	0.243	1.66
	S. thermophilus	0.1289	1.31	0.1462	1.54	0.1718	1.71	0.141	1.71
Lactose	*L. bulgaricus*	0.1141	1.64	0.142	1.88	0.2086	1.89	0.1418	1.66
	L. casei	0.1309	1.11	0.1864	1.49	0.2743	1.8	0.1418	1.71
	L. lactis	0.1036	1.6	0.1219	1.75	0.171	1.91	0.1019	1.85
	S. thermophilus	0.1032	1.41	0.1273	1.58	0.1486	1.89	0.1118	1.8
Sorbitol	*L. bulgaricus*	0.083	1.29	0.1233	1.74	0.1587	1.9	0.133	1.9
	L. casei	0.064	1.27	0.0709	1.3	0.1218	1.52	0.1025	1.38
	L. lactis	0.021	0.77	0.0936	1.56	0.1225	1.85	0.1158	1.66
	S. thermophilus	0.0417	0.87	0.0663	1	0.1284	0.7	0.1133	0.53
Starch	*L. bulgaricus*	0.042	1.08	0.0709	1.45	0.0986	1.82	0.0553	1.48
	L. casei	0.0519	1.24	0.0702	1.72	0.1014	1.86	0.0737	1.83
	L. lactis	0.0516	1.08	0.0809	1.54	0.101	1.87	0.068	1.84
	S. thermophiles	0.0245	1.39	0.076	1.39	0.1094	1.87	0.0945	1.67

Table 5. Influence of nitrogen sources on growth and hydrogen peroxide production by the test isolates.

N_2 sources		Incubation period (h)							
		12		24		48		72	
		H_2O_2 produced		H_2O_2 produced		H_2O_2 produced		H_2O_2 produced	
	Test isolates	(mg/L)	Growth (O.D)	(mg/L)	Growth (O.D)	(mg/L)	Growth (O.D)	(mg/L)	Growth (O.D)
Yeast extract	*L. bulgaricus*	0.1473	1.83	0.1608	1.86	0.1892	1.88	0.1664	1.88
	L. casei	0.1248	1.76	0.1546	1.89	0.1895	1.89	0.1663	1.86
	L. lactis	0.1405	1.55	0.1532	1.79	0.1846	1.82	0.1228	1.82
	S. thermophilus	0.1073	1.37	0.1271	1.75	0.1698	1.88	0.1418	1.81
$(NH_4)_2SO_4$	*L. bulgaricus*	0.0266	0.39	0.0448	0.87	0.1013	1.06	0.0354	0.92
	L. casei	0.0315	0.25	0.0711	0.68	0.0945	1.15	0.0545	1.05
	L. Lactis	0.0231	0.23	0.0448	0.44	0.0711	1.05	0.0474	0.88
	S. thermophilus	0.0304	0.19	0.0711	0.51	0.1308	1.08	0.0887	0.64
Urea	*L. bulgaricus*	0.0397	0.72	0.0475	0.84	0.0679	0.95	0.0538	0.83
	L. casei	0.0189	0.43	0.0297	0.78	0.0337	0.89	0.0189	0.78
	L. lactis	0.0432	0.62	0.0837	0.74	0.1213	1.15	0.0914	0.76
	S. thermophilus	0.0242	0.39	0.0536	0.74	0.0769	0.94	0.0417	0.78
Casein	*L. bulgaricus*	0.1335	1.66	0.1564	1.81	0.1833	1.87	0.1632	1.84
	L. casei	0.1041	1.32	0.1261	1.4	0.1827	1.9	0.1413	1.87
	L. lactis	0.1289	1.58	0.1341	1.58	0.1648	1.88	0.1104	1.79
	S. thermophilus	0.0382	1.18	0.1219	1.3	0.1799	1.85	0.1416	1.8

Table 6. Inhibitory activity of hydrogen peroxide- fluid produced by some LAB against indicator organisms.

LAB isolates	Target organisms		
	***C. albicans* (mm)**	***E. coli* (mm)**	***S. aureus* (mm)**
L. bulgaricus	---	21.0	14.0
L. casei	---	16.0	18.0
L. Lactis	---	16.0	17.5
S. thermophilus	---	17.0	17.3

--- = no inhibition zone, 0.0 – 5.0 mm = very weak, 6.0 – 10.0 mm = weak inhibition,11.0 – 15.0 mm = slightly strong inhibition, 16.0 – 20.0 mm = strong inhibition, 21.0 – 25.0 mm= very strong inhibition.

other carbon sources used in this work. The LAB isolates showed an increase in growth and hydrogen peroxide concentration as incubation period increases which later decreased at the end of 72 h incubation period. This could be as a result of the ability of lactic acid bacteria to metabolize different carbon sources differently which is based on the specific activities involved in carbohydrate degradation. Lactobacilli may be able to grow, produce and accumulate hydrogen peroxide under aerobic condition in a glucose and galactose medium (Whittenbury, 1964; Gregory and Fridovich, 1974). Rebecca et al. (2008) concluded that variation in the concentration of constituents or supplements of cultivated media might have an influence on the amount of antimicrobials production by microorganisms.

All the isolates preferred yeast extract as nitrogen source for hydrogen peroxide production compared to tri-ammonium sulphate used in this work. Lactic acid bacteria required complex nitrogen sources, which is very important in hydrogen peroxide production and for growth (Suma et al., 1999). This is in contrast to the report of Adesokan et al. (2010) who detected lowest amount of hydrogen peroxide when yeast extract was employed as a nitrogen source.

Hydrogen peroxide produced by the lactic acid bacteria isolates showed inhibitory activity against *E. coli* K12 and *S. aureus* as indicator organisms in this work. This observation is in agreement with the findings of

Ogunbanwo et al. (2004) and Ito et al. (2003). The bactericidal effect of hydrogen peroxide has been attributed to its strong oxidizing effect on the bacterial cells and to the destruction of basic molecular structure of the cell protein (Zalan et al., 2005). In the study of Bernet-camardm et al. (1997), Hudault et al. (1997), Sgouras et al. (2004), Lactobacilli that exhibit adhesive properties were shown to have strong inhibitory activity against invasive pathogens such as *Vibrio, Shigella, Salmonella* and *Helicobacter.*

Conclusion

Lactic acid bacteria may be generally regarded as safe for preservation of dairy products due to the production of hydrogen peroxide. The isolated LAB isolates in this study produced hydrogen peroxide at different temperature, pH, carbon and nitrogen sources with the optimum production at 37°C and acidic pH of 5.5 in modified MRS broth containing 2% galactose and 2% yeast extract. It was shown that LAB isolated from raw and fermented milk in South-West Nigeria are capable of producing hydrogen peroxide which has antagonistic effect on pathogenic organisms. *E. coli* was the most sensitive to H_2O_2 among the species examined. The high sensitivity of *E. coli* to H_2O_2 was also demonstrated by Ito et al. (2003).

In the USA, the use of H_2O_2 as an oxidizing or bleaching agent for wine, tea, tripe, herring, beef feet, dried eggs, and as a GRAS substance in whey (0.04%), starch (0.15%), milk (0.05%), corn syrup (0.15%) and other foods is permitted (Sofos and Busta, 1999). Therefore, the addition of live cells of H_2O_2-producing LAB as starter culture or as an adjunct starter will not only contribute to the flavor and aroma of yoghurt and other dairy products but will be useful for food preservation as well as prevention of growth of food-borne pathogens.

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