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REGULAR ARTICLE

CONVERSION OF MALIC ACID IN TO LACTIC ACID IN ALOEVERA BY USING LACTIC ACID BACTERIA

Avinash Tungala[1*], Ajay J.Y[2], Pradeep Kumar Gajula[2], Dinesh. J[1], Deepak kumar. J[3]

[1]*Department of Industrial Biotechnology, School of Chemical and Biotechnology, SASTRA University, Tanjavur – 613401*
[2]*Department of Pharmaceutical Technology, School of Chemical and Biotechnology, SASTRA University, Tanjavur – 613401*
[3]*Department of Biotechnology, SRM University, Chennai*

SUMMARY

Malic acid is an excellent indicator of gel freshness. This acid is produced naturally in the leaves of aloes and other succulents whose cells contain large, water-filled vacuoles. Under poor handling conditions in the presence of bacteria, malic acid can be broken down to form lactic acid. Gram-negative rod bacteria, which grow in some aloe gels, assimilate malic acid and free glucose and produce other organic acids such as lactate or lactic acid. Lactic acid is one of the major products of carbohydrate break-down by lactic acid bacteria during malolactic fermentation (MLF). Malolactic enzyme demonstrated to be present in most lactic acid bacteria but not in other bacteria, catalyzes the reaction:

1 malate + H+-1lactate+Co_2.

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*Corresponding Author, Email: avinash.tungala9@gmail.com

1. Introduction

Aloe vera, also known as the Medicinal Aloe, is a species of succulent plant that probably originated in northern Africa. The species does not have any naturally occurring populations, although closely related Aloes do occur in northern Africa. *Aloe vera* grows in tropical climates and is widely distributed in Africa, Asia and other tropical areas. The species is frequently cited as being used in herbal medicine. Extracts from *A. vera* are widely used in cosmetics and alternative medicine, being marketed as having rejuvenating, healing, and soothing properties. There have been many scientific studies of the use aloe Vera, some of it conflicting. Despite these limitations, there is some preliminary evidence that *A. Vera* extracts may be useful in the treatment of diabetes and elevated blood lipids in humans. These positive effects are thought to be due to the presence of compounds such as polysaccharides, mannans, anthraquinones and lectins. *A. Vera* is a stemless or very short-stemmed succulent plant growing to 60–100 cm (24–39 in) tall, spreading by offsets.

Fig 1. *Aloe vera*

Scientific classification in *Aloe vera*:
Kingdom: Plantae
Family : Liliaceae
Genus : Aloe
Species : Barbadensis

The stems, thick and fleshy, green to grey-green, with some varieties showing white flecks on the upper and lower stem surfaces. The margin of the stem is serrated and has small white teeth. The flowers are produced in summer on a spike up to 90 cm

(35 in) tall, each flower pendulous, with a yellow tubular corolla 2–3 cm (0.8–1.2 in) long. Like other *Aloe* species, *A. Vera* forms arbuscular mycorrhiza, a symbiosis that allows the plant better access to mineral nutrients in soil.

Chemical properties

Amino acids:

Provides 7 of 8 essential amino acids they are

Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Threonine, Valine

Amino acids like Aspartic acid, Glutamic acid, Alanine, Cystine,

Glycine , Histidine, Hydroxyproline, Proline, Serine and Tyrosine.

Enzymes

Provides 8 enzymes:

Aliase, Alkaline phosphatase, Amylase, Carboxypeptidases, Catalase, Cellulase, Lipase, Peroxidase.

Minerals

Calcium, Phosphorus, Potassium, Iron, Sodium, Chlorine, Manganese, Magnesium, Copper, Chromium, Zinc.

Monosaccharide and polysaccharides:

D-Arabinose, D-Glucose, D-Galactose, D-Mannose, L-Rhamnose, D-Xylose, Cellulase, Aldopentose, Acemannan .

Vitamins: Vitamin-A (Retinol),B1 (Thiamine), B2 (Riboflavin), Niacinamide,

Vitamin B6 (Biotin), Vitamin B12 (Cyanocobalamin),

Vitamin C (Ascorbic acid), Vitamin E (Tocopherol).

Aloe emodin

Aloe emodin is an anthraquinone present in aloe latex,

An exudate from the aloe plant. It has a strong stimulant-laxative action

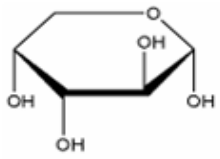
Free sugars

Carbohydrates are a group of organic compounds containing carbon, hydrogen and oxygen in the general proportions of 1:2:1.

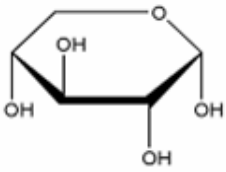
Monosaccharide can be defined as the simplest form of these carbohydrates. The analysis of mixtures of carbohydrates is important in various industries, especially those concerned with pharmacology and the preparation and stabilization of food and drink.

Examples of common monosaccharide are:

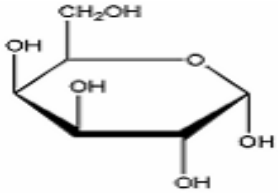
D-Aribinose



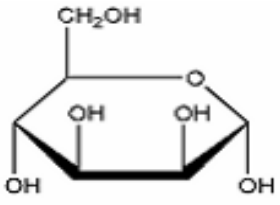
D-Glucose



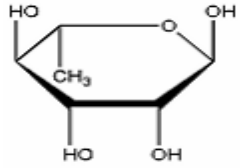
D-Galactose



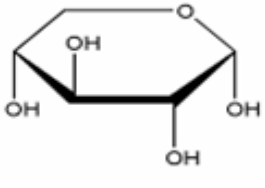
D-Manose



L-Rhmanose



D-Xylose



Aloin

Aloin, also known as Barbaloin [Reynolds, Aloes - The genus Aloe, 2004], is a bitter, yellow-brown colored compound noted in the exudates of at least 68 Aloe species at levels from 0.1 to 6.6% of leaf dry weight (making between 3% and 35% of the total exudates) [Groom & Reynolds, 1987), and in another 17 species at indeterminate levels [Reynolds, 1995b]. It is used as a stimulant-laxative, treating constipation by inducing bowel movements.

Aloe resin

It contains C-glycosides and resins, anthroquinone glycosides (including aloe-emodin and aloin A and aloin B).

Fatty acids helpful in tissue maturation

Linoleic, linolenic, myristic, caprylic, oleic, palmitic, stearic

Antiseptic

Cinnamic acid, lupeol (a natural salicylic acid), phenol, sulfur, urea nitrogen

Analgesic

Lupeol, magnesium (as lactate)

Anti-inflammatory

Brady kinase, B-sitosterol, campesterol, beneficial HDL cholesterol

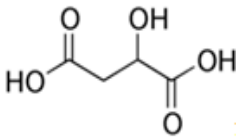
Natural defense system enhancers

A newly discovered component of the Aloe Vera plant, called acemannan, shows preliminary evidence of strengthening the body's natural defences. Alkaline phosphatase, creatine phosphatase, creatinine, glucose, lactate, sodium, triglycerides

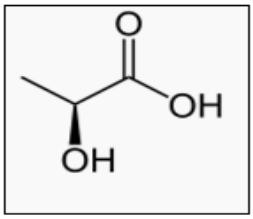
Malic acid:

Malic acid is an excellent indicator of gel freshness. This acid is produced naturally in the leaves of aloes and other succulents whose cells contain large , water filled vacuoles such plants have the crassulacean acid metabolism (CAM); an additional photosynthetic pathway where malic acid is produced naturally.

Malic acid



Lactic acid



Lactic acid (**2-hydroxypropanoic acid**), also known as **milk acid**, .

This form of chromatography requires that sugars be converted in to volatile derivatives such as alditol acetates (Hoebler et al., 1989).The derivatization in to the respective alditol acetates is favored as a single peak on a chromatogram as represents each alditol Opposed to trimethylsilyl derivatives, which generate multiple peaks.This is due to the fact that up to four different derivatives may be formed from a single monosaccharide as a result of anomeric and Ring isomerisation (sawardeker etal., 1965). All four chromatographic techniques confirmed that glucose is the only free sugar found in the gel of all the species of aloe investigated. The presence of glucose as practically the only free monosaccharide in Aloe Vera gel (Femenic et al., 1999 ; Christopher and holtum , 1996). Parameters that are routinely used in the evaluation and identification of commercial Aloe Vera gels are pH, malic acid and conductivity (Ni and Tizard,).

2. Materials and Methods

Washing glassware

Glassware were first soaked in chromic acid cleaning solution (10%Potassium dichromate solution 25%sulphuric acid) for few hours and washed. Thoroughly in tap-water after second washing in detergent solution they were again washed thoroughly in tap- water and rinsed in distilled water.

Glass were generally used is conical flasks, burette, pipette, test tubes, measuring cylinder.

Sterilization

Sterilization of culture media was carried out in an autoclave at 121°C for 15 minutes. The glassware was sterilized at 160°C in a hot air oven for 1 hr.

Collection of *Aloe vera* leaf:

In aseptic conditions Aloe Vera raw leaf was collected from the farm and from different places of Thanjavur.

Harvesting of sample

Aloe vera leaf was washed with distilled water and make extract in aseptic conditions. 1ml sample was collected and transferred it in to the FL broth (Fluid lactose broth) and kept incubation for 24 hrs at 32°C.FLB medium was prepared and Mix all the ingredients and autoclave for 15 minutes at 121°C. Checked its pH after sterilization the medium was Cool immediately. *Aloe vera* powder was collected in aseptic conditions. 5 gms of Aloe Vera powder was transferred in to the TSB broth (Tryptone Soya broth) and kept incubation for 24 hrs at 32°C. Leaves were harvested and filleted. Fresh aloe gel was used for the experimental work. Samples used for the individual parameter screening were left at room temperature for three days and then re-tested to detect possible changes associated with poor handling conditions.

Isolation of lactic acid bacteria by pour plate method

Lactobacillus was collected from Aloe Vera powder organism, Leaf surface organism.

Aloe Vera 1gm powder was taken and suspended with 50 ml TSB (Tryptone Soya broth) and from that 1ml was plated and added to lactobacillus MRS Agar medium and similarly to SDA medium. After adding to the medium the plates are kept for incubation for 24 hrs at 32°C.

Figure: 2 Cultural morphology of lacto bacilli in lacto bacilli agar medium

Identification of lactic acid bacteria by gram staining method

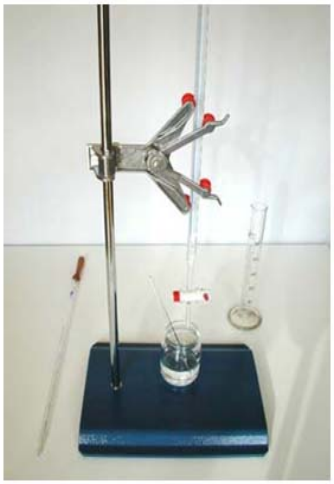
Lactobacillus can be identified by using gram staining technique. Petri plates with the colonies of micro organism are taken. Gram stain requirements, Glass slides, loop, microscope, burner Gram stain reagents crystal violet, grams iodine, grams decolorized solution and, safranin was required for staining. Glass slide is cleaned with the distilled water. With the sterile loop thin smear was made on the slide and then heat fixed the slide. Few drops of crystal violet is added to the smear. After 30-60 seconds slide is washed with distilled water then grams iodine is added to the slide and washed with water, following gram's decolorized solution and countered with safranin.

Identification of malic and lactic acid value in aloe vera by titremetric method

Aloe vera was collected in aseptic conditions and crushed at room temperature.50 ml of Aloe Vera extract was taken. This sample was titrated against 0.1N NaOH. By using phenolphthalein as indicator to find out the total acid concentration present in the solution.

Juice was kept for 3days at room temperature. And 50 ml of sample was taken titrated against 0.1N NaOH by using phenolphthalein as indicator.

Figure 3: Acid value estimation by titremetric method



Biochemical identification of lactic acid bacteria

Catalase test

Catalase test is used to identify the catalase producing micro-organisms. In this test H_20_2 is added to the culture to test the presence of catalase enzyme.

Acidic Determination (pH)

The pH of aloe gel was determined using a calibrated pH meter (crison micro pH 2000). The samples were once again analyzed fresh and after being stored for three days after being stored for three days at room temperature.

Quantification and identification of malic and lactic acid by hplc method

A Shimadzu LC-6AD HPLC system equipped with a Waters differential refract meter R401 was used. Fresh aloe gel was passed through a Cameo 0.22 mm nylon filter and 20 µl injected directly into the system. Reference samples of pure acids (4 mg/mL) were used as external standards. Peaks generated from the aloe gel were identified by comparison of their retention times and co-injections. Concentrations of organic acids were calculated using peak areas generated from the simultaneous analysis of organic acids and sugars. Samples were analyzed fresh (directly from the fridge) and once again three days after being kept at room temperature.

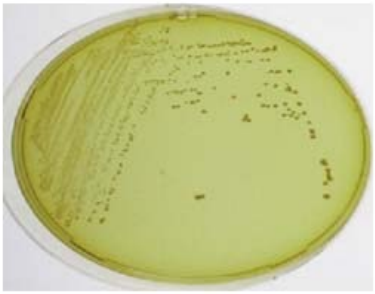
3. Result

Morphological characters of lactic acid bacteria in different media

Morphological characters of lactic acid bacteria on MRS Agar medium:

After incubation of 72 hrs at 32°C Lacto bacilli appear as large, white pointed colonies on the surface of the Agar medium was observed.

Figure 4 Lacto bacilli in agar medium



Morphological characters of lactic acid bacteria on TSA medium:

Large, spreaded, irregular, white colored colonies are observed on tryptone Soya agar medium.

Morphological characters of lactic acid bacteria on Tomato Juice Agar medium:

Colonies are large, spreaded, irregular colonies are observed. Cultural characters was observed after 40-48 hrs at 35 ± 2°C.

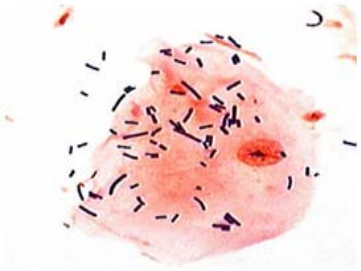
Morphological characters of lactic acid bacteria on Fluid Lactose broth:

Growth in the broth medium is indicated by the presence of Turbidity compared to an un-inoculated control after 48 hrs incubation at 32°C.

Microscopic observation

In gram staining violet colored, rod shaped bacteria was observed, by this it is conformed as gram-positive bacillus.

Fig.2 Gram staining



Malic and lactic conversion in fresh aloevera gel and three days older gel

By the effect of lactic acid bacteria malate converting to lactate. By this Lactic acid concentration was increased at 0.40% in three days old gel.

Table: 1 Malic and lactic acid levels in fresh and three days old gel

Aloe species	Fresh aloe gel		Three day old aloe gel stored at room temperature	
	Malic acid (mg/ml)	Lactic acid (mg/ml)	Malic acid (mg/ml)	Lactic acid (mg/ml)
A.barbadensis	0.71	0.96	0.151	1.546

pH

In fresh Aloe Vera gel and after 3 days old gel shows difference in old Aloe Vera gel. pH was decreased to 4.81, and in fresh gel pH was observed as 5.6.

Table:2 pH levels in aloe gels and changes associated with storage

Aloe species	Acidity(pH)	
	Fresh Extract	3 days at room temparature
A.barbadensis	5.6	4.81

Bio chemical identification

Lacto bacillus was confirmed by its catalase reaction in catalase test bacteria possess catalase that is catalase positive.

HPLC method for quantification and identification of malic and lactic acid

Powder organisms

These are the gram positive bacillus bacteria which are observed in Aloe Vera Powder and Identified as Lactobacillus.

Leaf surface organisms

These are gram negative bacillus bacteria observed on the surface of the gel.

Malic and Lactic acid

Table:3 Malic and Lactic acid

	Malic acid	Lactic acid
Powder organisms	15.82mg/g	30.80 mg/g
Leaf surface organisms	9.50 mg/g	26.38 mg/g

Table 4 Malic and lactic acid levels in powder sample(89 + 90 a) at 24 and 48 hrs

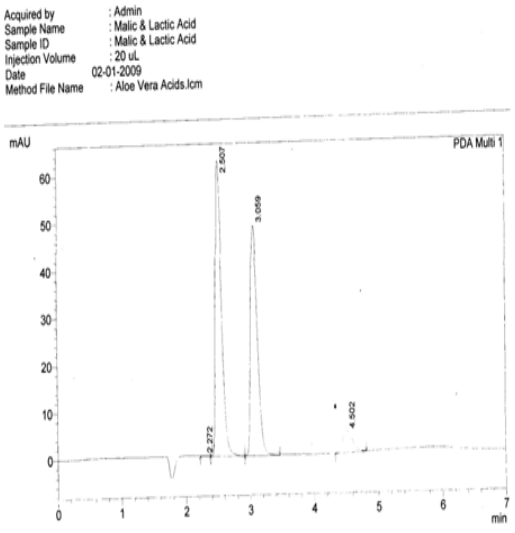
Medium / organism	Powder Sample (89+90A)	
	24 hrs	48 hrs
DM WATER BLANK	M.A.= 0.21	M.A.= 0.23
CONTROL	L.A.= 0.29	L.A.= O.30
DM WATER BLANK	M.A.= 0.13	M.A.= 0.21
TEST	L.A.= 0.18	L.A.= 0.29
LEAF SURFACE ORGANISM	M.A.=0.20	M.A.= 0.21
CONTROL	L.A.= 0.27	L.A.= 0.29
LEAF SURFACE ORGANISM	M.A.= 0.06	M.A.= 0.09
TEST	L.A.= 0.09	L.A= 0.12
POWDER ORGANISM	M.A.= 0.20	M.A.= 0.21
CONTROL	L.A.= 0.27	L.A.= 0.29
POWDER ORGANISM TEST	M.A.= 0.17	M.A.= 0.06
	L.A.= 0.23	L.A.= 0.09

*M.A – Malic acid ; L.A- Lactic acid

Table 5 Malic and lactic acid levels in powder sample(89 + 90 b) at 24 and 48 hrs

Medium / organism	Powder Sample (89+90B)	
	24 hrs	48 hrs
DM WATER BLANK	M.A.= 0.32	M.A.= 0.32
CONTROL	L.A.= 0.43	L.A.= O.43
DM WATER BLANK	M.A.= 0.27	M.A.= 0.29
TEST	L.A.= 0.36	L.A.= 0.40
LEAF SURFACE ORGANISM	M.A.=0.29	M.A.= 0.29
CONTROL	L.A.= 0.40	L.A.= 0.40
LEAF SURFACE ORGANISM	M.A.= 0.16	M.A.= 0.23
TEST	L.A.= 0.21	L.A= 0.30
POWDER ORGANISM	M.A.= 2.25	M.A.= 0.29
CONTROL	L.A.= 4.34	L.A.= 4.40
POWDER ORGANISM TEST	M.A.= 2.47	M.A.= 2.08
	L.A.= 6.63	L.A.= 6.10

*M.A – Malic acid ; L.A- Lactic acid

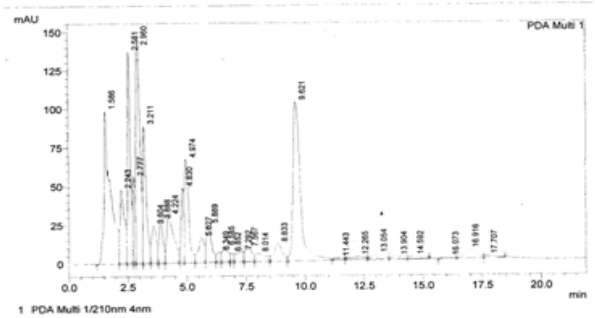


PeakTable

PDA Ch1 210nm 4nm

Peak#		Ret. Time	Area	Height	Area %	Theoretical Plate#	Tailing Factor
1		2.272	119	33	0.014	4269.593	1.330
2	MA -	2.507	411520	62302	49.902	2869.906	1.579
3	LA -	3.059	364320	48633	44.178	3401.820	1.523
4		4.502	48702	4548	5.906	4028.394	1.460
Total			824662	115515	100.000		

Acquired by : Admin
Sample Name : Aloe Vera
Sample ID : Leaf organisum
Injection Volume : 20 uL
Data : 02-01-09
Method File Name : Aloe Vera Acids.lcm



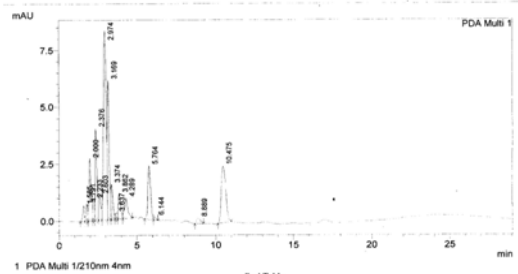
1 PDA Multi 1/210nm 4nm

PeakTable

PDA Ch1 210nm 4nm

Peak#		Ret. Time	Area	Height	Area %	Theoretical Plate#	Tailing Factor
1		1.586	1936821	98583	13.961	445.831	0.000
2		2.243	624334	47553	4.500	536.862	0.000
3	MA -	2.581	1145687	136808	8.259	1891.575	0.000
4		2.777	348638	56157	2.513	472.434	0.000
5	LA -	2.960	1760645	146968	12.691	1094.970	0.000
6		3.211	862910	88526	6.220	1952.688	0.000
7		3.604	351614	24374	2.535	821.332	0.000
8		3.888	321347	27793	2.316	2065.404	0.000
9		4.224	664955	30400	4.793	781.433	0.000
10		4.830	392797	48235	2.831	391.528	0.000
11		4.974	822645	66986	5.930	2014.412	0.000
12		5.627	276355	15949	1.992	1372.323	0.000
13		5.889	370706	24120	2.672	2963.700	0.000
14		6.349	78557	6410	0.566	179.924	0.000
15		6.585	166168	9750	1.198	1709.930	0.000
16		6.852	58750	5611	0.423	53.844	0.000
17		7.292	143463	6771	1.034	41.341	0.000
18		7.567	186724	8773	1.346	1184.691	0.000
19		8.014	161830	5044	1.167	133.905	0.000
20		8.833	272308	11291	1.963	3656.509	0.000
21		9.621	2428227	102560	17.504	5791.179	1.905
22		11.443	8607	596	0.062	3012.329	0.000
23		12.265	45452	1302	0.328	1612.026	0.000
24		13.054	94356	2859	0.680	4053.154	0.000
25		13.904	51188	1284	0.369	1229.962	0.000

Acquired by : Admin
Sample Name : Aloe Vera Juice
Sample ID : Powder organisum
Injection Volume : 20 uL
Data : 02-01-09
Method File Name : Aloe Vera Acids.lcm



1 PDA Multi 1/210nm 4nm

PeakTable

PDA Ch1 210nm 4nm

Peak#		Ret. Time	Area	Height	Area %	Theoretical Plate#	Tailing Factor
1		1.585	4689	677	1.301	1108.716	0.000
2		1.791	5169	762	1.435	1048.339	0.000
3		2.000	27013	2751	7.497	919.904	0.000
4		2.233	6647	929	1.845	113.986	0.000
5		2.376	32556	4023	9.035	1649.798	0.000
6	MA -	2.603	10099	1094	2.803	284.303	0.000
7		2.974	100445	8297	27.877	1121.378	0.000
8	LA -	3.169	50836	6097	14.109	2067.620	0.000
9		3.374	12664	1534	3.515	1056.313	0.000
10		3.637	2497	267	0.693	322.241	0.000
11		3.862	11713	1082	3.251	2731.801	0.000
12		4.289	12957	882	3.596	2593.089	0.000
13		5.764	28393	2352	7.880	5287.858	1.105
14		6.144	1317	137	0.365	4054.014	0.000
15		8.889	3846	229	1.067	5117.747	1.157
16		10.475	49475	2477	13.731	6405.584	1.302
Total			360316	33589	100.000		

4. Discussion

There is a change in pH after the aloe gel has been stored at room temperature for three days . However, the pH values do not exhibit a general increase or decrease but rather erratic changes. Lactic acid formation is associated with ageing of aloe gel, resulting in a concentration decline of malic acid levels caused by bacterial infection. A possible explanation for this relationship is that glucose (free from) can also be converted in to lactic acid which can result in an increase of free ions or conductivity with in decaying aloe gels. Increases in pH values are expected, as lactic acid is a weaker acid than malic acid . The degradation of malic acid during MLF to lactic acid results in an increase in pH. Some species studied showed the 'expected' increase in pH, but some showed a decrease in pH.

5. Conclusion

The lactic acid bacteria have efficiency to convert the malate to lactate in 72 hrs incubation time.

The result indicates that the amount of malate to lactate conversion was same as 1 malate to 1 lactate and producing CO_2. The amount of malic and lactic conversion was depending on the Incubation time. Malo lactate conversion was high in 72 hrs at 32°C. Malic acid in Aloe Vera gel determined the freshness of the gel. Lactate was produced by Lactic acid bacteria in normal conditions.

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