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Cost-effective fermentative production of calcium lactate using BISS (below Indian standard sugar) and *Spirulina* hydrolysate

A K Yadav, A B Chaudhari and R M Kothari[1]*

School of Life Sciences, North Maharashtra University, Jalgaon 425 001, India
[1]Jain Hi-Tech Agri-Institute, Jain Irrigation Systems Ltd., Jalgaon 425 001, India

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Batch fermentation of BISS (below Indian standard sugar) in combination with *Spirulina* hydrolysate (SH) has been successfully explored at 50 L scale to replace costly glucose and yeast extract (YE) medium as a source of complex nutrients to produce calcium lactate economically by using *Lactobacillus delbruckii*. This has led to optimization of a protocol for cost-effective production of calcium lactate. Reduction in duration of fermentation has potential to provide additional benefits of (i) more batches per unit capital investment and time, (ii) less consumption of utilities, and (iii) higher output per unit labour cost.

Keywords: *Lactobacillus delbruckii*, BISS, calcium lactate, *Spirulina* hydrolysate

Introduction

Calcium lactate is widely used as a food preservative, feed supplement, pharmaceutical as a calcium replenisher, and an intermediate for the production of biodegradable plastics and other "green" compounds[1]. The market research revealed existence of vast scope for its large scale utilization, if the cost of its production could be reduced. As per one estimate, the raw materials alone account for 45-60% cost of lactate production[2]. Therefore, there have been continued efforts to explore economical alternative substrate(s) to meet carbon needs for microbial growth and complex nutrient source(s) to meet physiological and production needs to reduce the cost of production[3-6]. Towards these objectives, focus was accorded to replace costly ingredients, like glucose, sucrose and lactose by the hydrolysate of starchy grains or other sugars[7]. Similarly, replacement of costly yeast extract, peptone, etc was sought to meet complicated nutritional requirements of lactic acid secreting bacteria[8]. Most of the lactobacilli required growth nutrients like amino acids, vitamins, fatty acids, micronutrients and pure substrate(s) for their growth and lactic acid production[9]. Recent studies have indicated that supply of complex nutrients in the medium enhanced the productivity of the calcium lactate/lactic acid[10]. All these observations implied an obvious need for alternative renewable economic ingredients.

On this background, in the present study, use of an economical substrate sugar dust (known as Below Indian Standard Sugar, BISS), generated during the production of cane sugar, and for complex nutrients, hydrolysate of blue green alga (*Spirulina*) are explored.

Materials and Methods

Materials

BISS and sucrose were procured from KCP Sugar and Industrial Corporation Ltd., Vuyyuru, Andhra Pradesh. The yeast extract (YE) was obtained from Chaitanya Biological Pvt. Ltd., Buldana, Maharashtra and *Spirulina* from HDV Chem-Pharma (I) Pvt. Ltd., Chennai. Analytical Reagents were procured from S.d. Fine Chemicals, Mumbai.

Spirulina Hydrolysate

Spirulina hydrolysate (SH) was prepared by the standard method[11-12]. It is an amber coloured aqueous solution with 65% dissolved solids, 10.8% total nitrogen and 3% amino nitrogen.

Inoculum Preparation

Lactobacillus delbruckii isolated from a local dairy was sub-cultured and preserved on MRS medium[13]. Inoculum for lab scale and scale-up studies was prepared in 5 L shake flasks using MRS medium (37±1°C, 24 h) and acclimatized for economical

*Author for correspondence:
Tel: 91-257-2260011/22; Fax: 91-257-2261111/22
E-mail: agripark@jains.com

production at 42°C in a shaker incubator (Orbitech, 150 rpm, 24 h).

Experimental Set Up for Lab Scale Studies

Lab scale (1 L) studies were conducted using a pre-optimized control medium comprised of sucrose (5%), YE (1%), ammonium nitrate (0.5%) and malt extract (0.1%), *p*H 6.8±0.1. The experimental media comprised of BISS (5%) as sucrose substitute and SH as YE substitute. These were sterilized (121°C, 30 min), cooled and inoculated with *L. delbruckii* inoculum (24 h old, 5% v/v, 7×10^6 cells/mL, *p*H 4.95) and fermentation carried out on an incubator shaker (Orbitech, 44±1°C, 150 rpm, 48 h). The *p*H of fermentation media was maintained at 5.5±0.1 by adding pre-sterilized required amount of 20% lime slurry. The following fermentation parameters were monitored and their values noted.

Analytical Methods for Monitoring Fermentation Parameters

The *p*H was monitored using digital *p*H-meter (Elico). Microscopic examination of *L. delbruckii* growth and its homogeneity was monitored by using simple microscope (Nikon). The cell count was performed using cell counter (Lapiz). Lactic acid was routinely estimated as per IP (1996)[14-16] rather than by HPLC or colorimetry; the method being simple, rapid, cost-effective and desirable for Indian Pharmacopeia (IP) grade lactic acid as well as for reliable results in the hands of operators working round the clock.

Scale-up Production of Lactic Acid

In a typical experimental set up, 2.5 kg BISS and 400 g *Spirulina* hydrolysate were taken in 40 L de-mineralized (DM) water and fortified it with 250 gm ammonium nitrate, 50 g malt extract and *p*H adjusted to 6.8±0.2. It was transferred to stainless steel jacketed fermenter (length 100 cm, internal diameter 30 cm, L/D ratio 3.3, 4 mm thickness; 4 baffles, each 30 cm in length and 6 cm in width), having sample collection valve, air vent, inlet for lime addition and peeping window and fitted with digital temperature indicator, an agitator with two impellers with tip speed of 150 rpm. The medium was sterilized (121°C, 15 lbs pressure, 30 min), cooled to 48°C and inoculated [10% (v/v) 24 h old culture ($1.2\text{-}1.5\times10^6$ cells /mL)] under aseptic condition and fermentation carried out at 48±1°C, 150 rpm and *p*H 5.5±0.1. The *p*H was maintained by addition of pre-sterilized hydrated lime to neutralize lactic acid and carry forward the reaction for optimum conversion of sugar into calcium lactate. Fermentation parameters were monitored as earlier as 12 h interval. Similar 3 scaled up batches (batch size 40 L) were carried out.

Results and Discussion

Commercial Availability and Properties of BISS

BISS, a fine sucrose powder, carrying suspended particulate matter, has no attractive sale value, being unfit for human consumption. Its recycling is also not economical as it consumes more chemicals and gives less recovery, rendering it commercially least attractive. Typical analysis of BISS is summarized in Table 1.

In the absence of any commercial alternative, an effort has been made to use BISS and convert it into calcium lactate by providing a cheaper (Rs. 7-9/kg) carbon source to replace sucrose (Rs. 15-17/kg). While BISS is commercially available at Rs. 8000±1000 per ton, its cost goes up when the cost of sugar goes up. However, the price difference is substantial between these two commodities. Using (a) BISS to substitute sucrose and (b) SH to substitute YE, lactic acid production was carried out at laboratory scale. Its results are summarized in Table 2.

Substitution of Sucrose with BISS without Compromising Productivity

From Table 2, it is clear that, compared to control medium (pre-optimized during initial studies), the experimental medium also exhibited comparable (i) *p*H and growth profile, (ii) shape of *L. delbruckii*, (iii) cell count, (iv) % lactic acid secretion, and (v) % residual reducing sugar. Since all ingredients of both media were identical in nature and concentration, except the source of carbon (sucrose in control medium substituted by BISS in the Experimental medium 1), it was an unequivocal proof that sucrose could be substituted by BISS without compromising

Table 1—Typical analysis of BISS

Parameters	Results
Total sugar (%)	98-99
Reducing sugar (%)	0.10-0.15
Particle size (mesh)	100-200
Suspended particulate matter (%)	0.1
Moisture (%)	1.1-1.5
Appearance of solution (15% w/v)	Soluble, hazy, slightly yellowish with particulate matter

Table 2—Profiles of microbial growth, *p*H and lactic acid production as a function of change in the source of carbon and nitrogen

No.	Parameter monitored	Control medium		Experimental medium 1		Experimental medium 2	
		24 h	48 h	24 h	48 h	24 h	48 h
1	*p*H	5.32	4.46	5.34	4.49	5.35	4.25
2	Microbial growth	More	Excellent	More	Excellent	More	Excellent
3	Shape of microbes	Long rods	Long rods	Long rods	Long rods	Long rods	Long rods
4	Cell count (10^7)	3.5	6.5	3.6	6.5	4.0	7.6
5	Lactic acid %	0.85	1.57	0.85	1.56	1.0	1.78
6	Reducing sugar %	4.65	3.55	4.28	3.51	4.35	2.89

Control medium = 5% sucrose, 1% YE, 0.5% ammonium nitrate and 0.1% malt extract; Experimental medium 1 = 5% BISS in place of 5% sucrose, retaining other ingredients in control medium; Experimental medium 2 = 5% BISS+1% SH, retaining 0.5% ammonium nitrate and 0.1% malt extract present in the control medium. Experimental medium 1 shows that 5% sucrose is substitutable by 5% BISS, while Experimental medium 2 shows that 1% YE is substitutable by 1% SH; Growth = no. of cells in 1 mm^2 area under the microscope.

the growth of the culture as well as its productivity of lactic acid. This observation encouraged us to explore the substitution of YE by SH. Therefore, it was of interest to find out the composition of both YE and SH, which may shed some light on substitutability of the YE by SH.

Comparative Analysis of *Spirulina* Hydrolysate *vis-à-vis* Yeast Extract

Table 3 has summarized analytical profiles of SH as well as YE. It has shown broad comparability in % proteins, carbohydrates, lipids and ash content. SH has several pigments, while YE is free from them. While their strict comparability is difficult, their contribution to lactic acid production appears worth pursuing, as metabolism permits substitution of one ingredient with another and, hence, many times apparently not strictly comparable inputs perform comparably through intermediary metabolism. Most significant attribute to note is that while YE is commercially available at Rs. 450-500/kg, SH can be produced at Rs. 300-350/kg and, hence, comparatively economical.

Substitution of YE with SH without Compromising Productivity

In the light of the above comparison, results of substitution of YE by SH are summarized in Experimental medium 2 (Table 2). Accordingly, it is clear that compared to the control medium, the experimental medium also exhibited comparable (i) *p*H and growth profile, (ii) shape of *L. delbruckii;* (iii) more cell count, (iv) % lactic acid secretion on higher side and (v) less % residual reducing sugar. All ingredients of both media were identical in nature and concentration; the only difference being substitution of sucrose by BISS and YE by SH. From higher productivity of lactic acid in Experimental medium 2 over Experimental medium 1, it appears that SH is equally effective as YE and results confirm that SH+BISS is superior to sucrose+YE. Thus, sucrose could be substituted by BISS without compromising the productivity of lactic acid and the same could also be said for substitution of YE by SH.

Baker's Yeast does not Substitute YE and *Spirulina* does not Substitute SH

At times, a protein is more effective than its hydrolysate as has been found in the fermentation of alkaline and acid proteases[17-18]. Therefore, *Spirulina* powder without hydrolyzing was used to know if it could substitute YE/SH. However, this effort did not succeed as it provided very poor growth of *L. delbruckii* and secretion of lactic acid was barely detectable. Although Baker's yeast upon enzymatic hydrolysis and spray drying gives YE, its use without enzymatic hydrolysis seems to give only nitrogen and not its growth promoting endogeneous constituents. Therefore, both *Spirulina* and Baker's yeast are no substitute to their respective hydrolysates as bio-availability of growth promoting ingredients in hydrolysate is more than without hydrolysis. Alternately, it was considered worthwhile to find out the reproducibility of lactic acid productivity as a function of SH at varying (0.2, 0.4, 0.6, 0.8 and 1%) concentrations. The growth of *L. delbruckii* as well as lactic acid productivity as a function of varying SH concentrations is summarized in Table 4.

Table 3—Analytical profiles of *Spirulina* hydrolysate and yeast extract[19]

No.	Content on dry basis	*Spirulina* hydrolysate	Yeast extract
	Protein %	70.0	60.2
	Carbohydrates %	16.5	12.9
1.	Lipids %	7.0	5.8
	Ash content %	9.0	5.6
	Moisture %	7.0	7.0
2.	Carotenoids	1.75	-
	Xanthophylls	1.18	-
	Chlorophyll-a	10. 15	-
	Phycocyanin	128.0	-
3.	Thiamine (B1)	26	14
	Riboflavin (B2)	34	35
	Niacin (B3)	148	420
	Pantothenic acid (B5)	6.0	140
	Pyridoxin (B6)	4.0	17.5
	Cyanocobalamin (B12)	0.7	0.3
	Biotin	0.3	0.5
	Folic acid	0.6	0.6
4.	Valine	60.0	36.9
	Isoleucine	41.3	-
	Leucine	58.0	42.3
	Lysine	40.0	41.4
	Methionine	21.7	7.20
	Phenylalanine	39.5	32.4
	Threonine	40.7	14.4
	Tryptophan	10.3	-
5.	Alanine	54.2	48.6
	Arginine	55.8	23.4
	Aspartic acid	60.3	48.6
	Cysteine	6.7	-
	Glutamic acid	80.4	93.6
	Glycine	34.6	21.6
	Histidine	10.8	10.8
	Proline	29.7	17.1
	Serine	35.0	15.3
	Tyrosine	31.4	-
	Magnesium	1.915	0.502
	Calcium	1.315	0.171
	Phosphorus	8.948	2.430
6.	Potassium	15.400	5.316
	Sodium	0.412	1.119
	Chloride	4.400	1.170
	Iron	0.580	-

Values of 3 are expressed as mg/kg, while that of 2, 4, 5 and 6 are expressed as g/kg. *Spirulina* is commercially available from Ballarpur Industries Ltd. (BILT, Nanjangud, Mysore). However, its hydrolysate was prepared and analyzed for its protein, carbohydrates, lipids, ash content and for vitamin/pigment contents. Its amino acid composition is taken from literature.

From Table 4, it is obvious that (i) medium 1 and 2 were comparable in all respects, (ii) medium 3 showed relatively higher *p*H, small rods of *L. delbruckii* and their less count, which all reflected in poor (0.95%) lactic acid production, indicating that more SH was needed for growth and production, and (iii) trend in medium 3 gradually showed improvement in medium 4-7, registering a reduction in *p*H, more microbial growth, higher cell count, long rods, increased lactic acid concentration as a function of increasing % SH. Optimal productivity of lactic acid was noted in medium 6, indicating that medium 1 and medium 2 could be effectively substituted by medium 6, representing 5% BISS and 0.8% SH. These observations being reproducible at shake flask level, there was no doubt that costly sucrose+YE of medium 1 could be replaced by economical BISS+SH.

From the studies on the utilization of BISS with SH, depicted in Fig. 1, it is apparent that (i) cell number increased substantially as a function of SH concentration in the medium, and (ii) maximum cell count was observed in the medium containing 0.8% SH.

Similarly, the trend of sugar conversion increased with the increase of SH in the medium (Fig. 2). Initially, the sugar in BISS decreased slowly (during the lag phase), then decreased rapidly (during the exponential growth phase) and finally decreased slowly (during the stationary and cell death phase). While at 0.2% SH, BISS has taken a duration of 144 h for optimal fermentation, reducing it to 120 h with 0.4% SH, reducing yet further to 96 h with 0.6% SH and finally completing the fermentation in 72 h with 0.8% SH. *A* similar trend has been reported with an increasing YE concentration (0.0 to 3.0 g/L) during the fermentation of lactose[20], a costly carbon source.

Effect of SH on Calcium Lactate Production

It is clear from Table 4 that calcium lactate production increased with an increasing dose of SH. At 0.2% SH, less production of calcium lactate was noticed. However, as % SH increased to 1%, the cell biomass also increased due to availability of higher amount of vitamins, macro- and micro-nutrients provided by SH, which in turn also resulted in maximum lactic acid formation, as judged from increasingly higher consumption of hydrated lime per unit time, giving maximum production of calcium lactate with 0.8% SH (Fig. 3). At scale-up studies, where 1.8 kg lactic acid was produced, consumption of lime was maximally 0.9-1.0 kg. Similar observations on other complex nitrogenous inputs have also been reported[20-22].

Table 4—Profiles of *L. delbruckii* growth *vis-à-vis* lactic acid production as a function of SH concentration

No.	Parameter	Medium 1	Medium 2	Medium 3	Medium 4	Medium 5	Medium 6	Medium 7
1	*p*H	4.47	4.49	5.29	4.94	4.76	4.35	4.32
2	Growth	More	More	Less	More	More	More	More
3	Shape of microbes	Long rods	Long rods	Small rods	Long rods	Long rods	Long rods	Long rods
4	Cell count (10^7)	6.4	6.5	5.5	6.9	6.7	7.5	7.6
5	Lactic acid %	1.55	1.56	0.95	0.98	1.45	1.95	1.92
6	Reducing sugar %	3.52	3.51	3.47	2.97	3.12	2.97	2.99

Medium 1 = 5% sucrose+1% YE; Medium 2 = 5% BISS+1% YE; Medium 3 = 5% BISS+0.2% SH; Medium 4 = 5% BISS+0.4% SH; Medium 5 = 5% BISS+0.6% SH; Medium 6 = 5% BISS+0.8% SH; Medium 7 = 5% BISS+1.0 % SH, retaining 0.5% ammonium nitrate and 0.1% malt extract in all media as in Table 2; Growth = no. of cells in 1mm^2 area under the microscope.

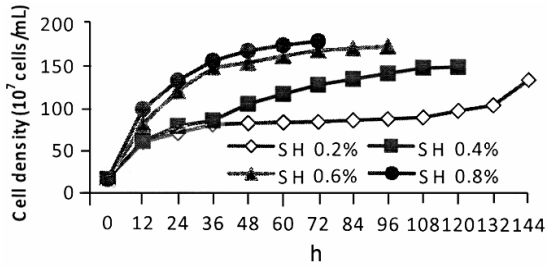

Fig. 1—Profiles of *L. delbruckii* growth as a function of *Spirulina* hydrolysate concentration

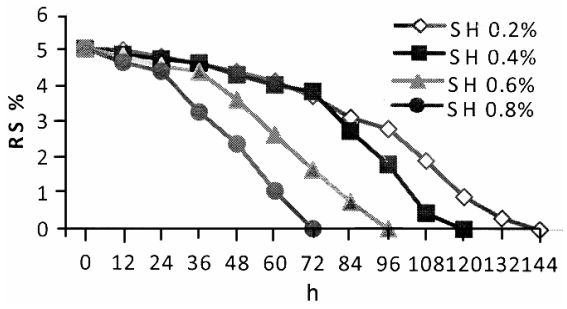

Fig. 2—Profiles of reducing sugars as a function of *Spirulina* hydrolysate concentration

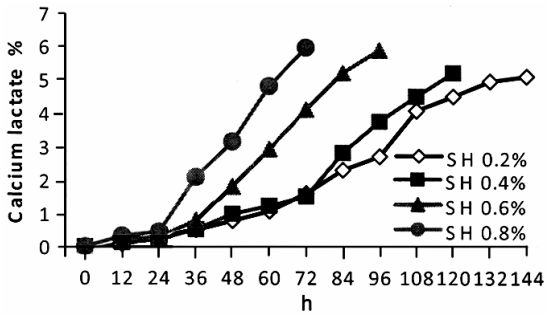

Fig. 3—Profiles of productivity of calcium lactate as a function of *Spirulina* hydrolysate concentration

Scale-up Studies

In the light of reproducible performance of 5% BISS+0.8% SH medium as judged from cell growth, reducing sugar consumption and calcium lactate production, fermentation was scaled up to 50 L so as to get a glimpse of (i) reproducibility on scale-up, (ii) difficulties during fermentation, (iii) difficulties during post-fermentation processing, and (iv) techno-economic feasibility for pilot scale (2000 L) production. The results of 3 batches of scale-up studies are summarized in Table 5.

Accordingly, it is clear from parameters monitored that scale-up from 1 to 50 L was without any difficulty. Similarly, no post-fermentation difficulties were experienced. Logically, therefore, the quality of calcium lactate was of commercial grade as with 5% sucrose+1% YE medium.

It may be noted that at shake flask fermentation, 44°C was optimum and fermentation terminated in 48 h. As against this, 48°C was optimal during scale-up. This is presumably related to geometry of the flask, its capacity for temperature dissipation and fermentation period. In scale up studies, temperature dissipation being superior, the culture performed well at 48°C and still required 69-74 h (Table 5) for complete fermentation. At 44°C, fermentation required 96-100 h for the same productivity and, hence, it was not preferred on larger scale to accommodate more number of batches per month.

Cost-effective Production Medium

It is well known that glucose is costly (Rs 20-25/kg). Therefore, a relatively cheaper carbon source was found in sucrose (Rs 15-17/kg). On this background, BISS is certainly the cheapest source of carbon (Rs.7-9/kg). No other source of carbon, such

Table 5—Profiles of microbial growth, *p*H and lactic acid production as a function of scale-up

No.	Parameter monitored	Batch 1	Batch 2	Batch 3
1	Terminal *p*H	5.80	5.70	5.85
2	Microbial growth	Excellent	Excellent	Excellent
3	Shape of microbes	Healthy long rod shape	Healthy long rod shape	Healthy long rod shape
4	Cell count (10^7)	4.80	4.90	4.70
5	Ferment termination (h)	69.0	70.0	74.0
6	Reducing sugar %	Nil	Nil	Nil
7	Calcium lactate %	5.95	6.05	5.88

Growth = no. of cells in $1mm^2$ area under the microscope.

as, starch hydrolysate or golden syrup (residue after glucose crystallization) could be cheaper than BISS. Thus, having selected most economical source of carbon for promoting the growth of *L. delbruckii,* attention was focused to find out economical substitute for costly YE (Rs 450-500/kg commercial grade). Soybean meal (SBM) is widely used as a complex growth promoting source of nitrogen. However, several researchers and the authors found that neither SBM nor any other de-oiled cake [sesame (*Sesamum indicum*), groundnut (*Arachis hypogaea*), coconut (*Cocos nucifera*), cotton seed (*Gossypium hirsutum*), sunflower seed (*Helianthus annuus*), rapeseed (*Brassica napus*), safflower seed (*Carthamus tinctorius*), jatropha seed (*Jatropha carcus*), mahua seed (*Madhuca indicum*), karanj seed (*Pongamia pinnata*)], whole wheat flour/whole wheat hydrolysate, whey permeates, etc] were found useful for the growth of *L. delbruckii* or secretion of lactic acid[23-24]. There have been a lot of effort to utilize other industrial byproducts to reduce the cost of production by replacing YE fully/partially. These efforts to reduce the cost, without compromising production, product quality and productivity did not succeed[23-27]. Certainly, the production cost could be reduced by utilizing molasses and corn steep liquor by fully replacing glucose, sucrose and yeast extract; however, it increased the cost of down-stream processing/purification, thereby limiting the use of calcium lactate/lactic acid. For the complete replacement of glucose/sucrose, BISS is cheaper (Rs 7-9/kg), abundant and always economically available. For complete replacement of YE, renewable SH [an ubiquitous micro-alga, cyano-bacterium or blue green alga (BGA), growing in tropical/subtropical climate in fresh water, seawater, brackish water and alkaline water; microscopically appearing filamentous spiral shaped organism], rich in protein, vitamins, minerals and several neutraceuticals is comparable. It is renewable and commercially available at a cheaper price (Rs 300-350/kg) in comparison with YE (Rs 450-500/kg). Together, use of BISS+SH costs Rs 38-40/kg of calcium lactate *vis-à-vis* Rs 48-50/kg by the established sucrose+YE medium.

Conclusion

From the foregoing studies, it is obvious that BISS and *Spirulina* hydrolysate (SH) can be effectively utilized for the optimized production of calcium lactate/lactic acid as an alternative cheaper source of sugar and complex nitrogenous growth promoting nutrients. At 5% BISS and 0.8% SH, more productivity of calcium lactate in lesser duration is observed due to presence of presumably higher amount of amino acids, vitamins and minerals as compared to yeast extract (YE). Further, no post-fermentation difficulties were encountered with the substitution of sucrose+YE medium by BISS+SH medium. This process has, therefore, potential to provide additional benefits of (i) more batches per unit capital investment and time, (ii) less consumption of utilities, and (iii) higher output per unit labour cost.

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