RESEARCH ARTICLE

Kavita R. Pandey
Babu V. Vakil

Development of bioprocess for high density cultivation yield the probiotic *Bacillus coagulans* and its spores

Authors' addresses:
Guru Nanak Institute for Research & Development (GNIRD), Guru Nanak Khalsa College, Opp. Don Bosco School, NP Marg, Mutanga 400019, Mumbai, India.

Correspondence:
Kavita R. Pandey
Guru Nanak Institute for Research & Development (GNIRD), Guru Nanak Khalsa College, Opp. Don Bosco School, NP Marg, Mutanga 400019, Mumbai, India.
Tel.: +91-2224096234
Fax: +91-2224016815
e-mail: kavita.v.dubey@gmail.com

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ABSTRACT

Bacillus coagulans is a spore forming lactic acid bacterium. Spore forming bacteria, have been extensively studied and commercialized as probiotics. Probiotics are produced by fermentation technology. There is a limitation to biomass produced by conventional modes of fermentation. With the great demand generated by range of probiotic products, biomass is becoming very valuable for several pharmaceutical, dairy and probiotic companies. Thus, there is a need to develop high cell density cultivation processes for enhanced biomass accumulation. The bioprocess development was carried out in 6.6 L bench top lab scale fermentor. Four different cultivation strategies were employed to develop a bioprocess for higher growth and sporulation efficiencies of probiotic *B. coagulans*. Batch fermentation of *B. coagulans* yielded 18 g L^{-1} biomass (as against 8.0 g L^{-1} productivity in shake flask) with 60% spore efficiency. Fed-batch cultivation was carried out for glucose, which yielded 25 g L^{-1} of biomass. C/N ratio was very crucial in achieving higher spore titres. Maximum biomass yield recorded was 30 g L^{-1}, corresponding to 3.8×10^{11} cells mL^{-1} with 81% of cells in sporulated stage. The yield represents increment of 85 times the productivity and 158 times the spore titres relative to the highest reported values for high density cultivation of *B. coagulans*.

Key words: *Bacillus coagulans*, high cell density fermentation, probiotics, process optimization, sporulation efficiency

Introduction

The quest for discovering how food can enhance health or prevent chronic diseases has triggered the research to study a wide range of components present in foods, besides the nutrients. Such foods are termed as functional foods. One such functional food are the probiotics. Probiotics have been defined by FAO/WHO as "Live microorganisms which when consumed in adequate amounts confer health benefits to the host" (Joint FAO/WHO report, 2007). Spore forming bacteria, primarily of the genus *Bacillus* and related genera, have been extensively studied and commercialized as probiotics (Sanders et al., 2008). *Bacillus coagulans*, the organism under study, is one such a Gram positive, spore forming lactic acid producing bacterium used by several leading probiotic companies.

Increasing productivity is the major objective of research in the fermentation industry as described by Lee et al. (1999) and Riesenberg & Guthke (1999). High cell density cultivation /fermentation (HCDC/ HCDF) enables the researchers to obtain far more quantity of biomass per run which can result in a higher product concentration. This kind of output is not achievable in conventional batch or continuous processes (Kleman & Strohl, 1994; Lee et al., 1999 Riesenberg & Guthke, 1999). What can be considered as a high cell density fermentation depends on the organism under study and it can range from 20 g L^{-1} to 200 g L^{-1} of dry cell weight (DCW) (Stratton, 1998; Choi et al., 2006).

HCDC was first carried out with yeasts for the production of single cell proteins, ethanol and biomass (Riesenberg & Guthke, 1999). The highest biomass recorded for HCDF of *Saccharomyces cerevisiae* and *E. coli* are 170 g L^{-1} and 190 g L^{-1} respectively (Chang et al., 1994; Shiloach & Fass, 2005). Probiotic bacterial fermentations have achieved cell titres ranging from 63 to 118 g L^{-1}. *Lactobacillus bulgaricus, L. cremoris* and *L. delbrueckii* produced 63, 88 and 118 g L^{-1} yields in terms of DCW, using different HCDC strategies (Schiraldi et al., 2003). Kamoshita et al. (1998), have reported productivity of 140 g L^{-1} DCW of *Lactococcus lactis* using HCDF strategies.

HCDC processes coupled with recombinant DNA technology have enabled proteins such as interferons, interleukins and growth hormones to be produced in huge quantities that might otherwise have been difficult, to obtain following conventional production methods (Bibal et al., 1991; Shiloach & Fass, 2005; Shojaosadati et al., 2008).

A large number of individual experiments are necessary for designing a fermentation process with the ultimate aim of commercialization (Zimmermann et al., 2006). Fed-batch processes are the most common strategies in industrial settings (Shilaoch & Rinas, 2010). One of the essential growth components, usually the C-source is added intermittently to the growing culture in a fed-batch process (Choi & Lee, 1999). Examples of fed-batch processes include antibiotics production, processes like ones employed for making penicillin, streptomycin, rifamycin (Birol et al., 2002; Hermann et al., 2003).

In the present research work a bioprocess for achieving HCDF for the probiotic culture *B. coagulans* was successfully developed. Different strategies like altered C/N ratio, mode of fermentation, enhanced sporulation efficiency and varying sugar concentrations, were adopted sequentially to arrive at a HCDF bioprocess.

Materials and Methods

All chemicals and reagents used for work reported in this chapter were procured from Merck India Ltd., or from SRL Chemicals, India. Media and media ingredients were obtained from HiMedia, India.

Culture and growth conditions

The probiotic strain was a gift from an Indian probiotic company. The inoculum were grown in inoculation medium (composition, g L^{-1}: dextrose – 5, peptone – 5, yeast extract – 5, $CaCl_2.2H_2O$ – 0.4, $MnSO_4.7H_2O$ – 0.28), and incubated on orbital shaker (Scigenics, Orbitek) at 37 °C at 150 rpm. Actual fermentations were carried out in production medium (composition, g L^{-1}: corn steep liquor (CSL) – 15, dextrose – 3, peptone – 0.5, $CaCl_2.2H_20$ – 0.37, $MnSO_4.7H_2O$ – 0.27). Each experimental set was carried out in triplicates.

Inoculum preparation. Saline suspension of bacterial cells was prepared using actively growing 18-20h old culture (A_{540nm} = 0.5) in seed medium (Dubey & Vakil, 2010). The A_{540nm} of the suspension was adjusted to 0.1 using sterile saline. Ten percent volumes of this suspension was aseptically transferred into 100 mL seed medium in 4 flasks of 500 mL capacity and incubated on orbital shaker at 37 °C. Details of inoculum preparation and quality parameters monitored are as listed in Table 1.

Fermentation conditions. Cultivation was carried out in a 6.6 L stirred tank bioreactor (Sartorius, Germany) with H/D ratio of 2.2:1, equipped with a pH electrode and a dissolved oxygen (DO) probe. The culture vessel was sterilized in an autoclave (PSM vertical autoclave, 98L capacity, Bengaluru) at 121 °C (15 psi) for 20 min. Ten percent inoculum was introduced into 4 L of fermentation medium. Agitation was provided by pair of 6 blade Rushton disc turbine impellers. To assess the cultivation process several parameters (discussed below) were monitored. Table 2 represents the process conditions employed for *B. coagulans* fermentation.

Table 1. *Seed preparation and quality parameters.*

Parameters	Details
Total volume	400 mL (100 mL medium in 4 × 500 mL Erlenmayer flasks)
Incubation conditions	37 °C, 250 rpm, 8-10 h
pH at transfer	6.5 ± 0.3
Culture morphology	Gram positive rods with sub-terminal spores
Inoculum volume	10%
Cell density at seed transfer	Corresponding to 0.5-07 × 10^8 CFU per mL^{-1}

Table 2. *Cultivation conditions for cultivation of B. coagulans fermentation.*

Inoculum volume	10% (400 mL)
Fermentation volume	4 L
Temperature	37 °C
pH	6.5 ± 0.1 at the time of inoculation
%DO	Maintained between 40-60% (±10%) till harvest
Agitator speed	250 rpm at the time of inoculation, and adjusted subsequently (range: 250-500 rpm) as necessary to maintain the DO% in the desired range
Aeration	0.5-1.0 VVM
Antifoam	30% silicone (v/v): added in minimum quantities to prevent and/or control foaming
Sampling frequency	0 h then every 2 h for determination of offline parameters
Batch duration	36-44 h

Process parameters. Parameters like temperature, pH, % DO saturation and agitation were monitored on-line while, parameters like DCW (method reported by Mills & Lee, 1996), residual glucose concentration (as reported by Sengupta et al., 2006), OD (A_{540nm}), pH and sporulation efficiency (as described by Dubey & Vakil, 2010) were recorded off-line. Additionally, batch sterility and morphology were checked under the microscope and by plating loopful of sample on nutrient agar (HiMedia, India Pvt. Ltd.) plates. Glucose estimation was done by 3, 5-dinitro salicylic acid (DNSA) method.

Batch trials. Total of 4 fermentation approaches were carried out with different strategies like lowering the C/N ratio, shift from batch to fed-batch mode of fermentation, enhanced spore titers etc. The aim was increasing the biomass to make the process a HCDF process.

Batch harvest. The decision to end the run was taken on the basis of observations for process parameters such as rise in %DO, sporulation efficiency (≥75%) and lower residual glucose concentration. Biomass yield (g L^{-1}) and sporulated fraction of the biomass were key factors taken into consideration to plan the next batch of fermentation.

Results

Batch trials

Batch 1. Aim of the first batch was to assess how the culture grows in a fermentor and how much biomass may be obtained before optimization. The C/N ratio of the fermentation medium was 40:1. The pH was not controlled during the run. Fermentation parameters for the batch are shown in Table 3.

Table 3. *Altered parameters for batch B1 of B. coagulans fermentation.*

Parameter	Details
C/N ratio	40:1
pH	6.5 ± 0.2
Batch duration	44 h
Mode of operation	Batch

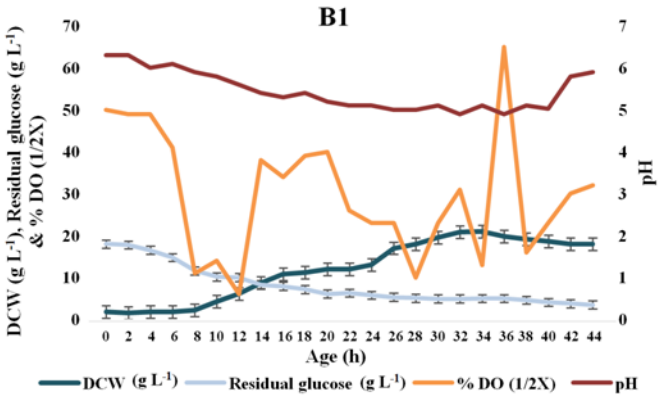

Figure 1. *Fed-batch cultivation profile of batch B1 for B. coagulans at C/N ratio 40:1.*

Figure 1 displays the trends of on-line and off-line parameters during the course of cultivation. Initial 8-12 h of process displayed a steep decline in %DO coupled with rapid increase in DCW suggesting that that the cells were actively multiplying in log phase. The DCW exhibited incremental trend in the first 24 h of growth. On extending the batch beyond 38 h the DCWs started declining so the batch was harvested at 44 h from the beginning of cultivation.

As can be noted from the graph above, pH values dropped from 6 to 4.5 between 12-26 h of fermentation. Around 28 h pH started increasing gradually and reached up to 5.5. This trend is indicative of diauxic growth pattern of *B. coagulans.* Lactic acid accumulation by the strain under active growth phase causes the drop in pH, while the rise in pH occurs due to utilization of lactates for metabolic activities.

Biomass (DCW) obtained from this batch (18.1 g L^{-1}, corresponding to 2.3×10^{11} cells per mL^{-1}) was significantly higher (2.25 times) compared to the productivity (DCW) under optimized shake flask conditions (8.0 g L^{-1}). It can be assumed that biomass production was highly improved because of better aeration and agitation conditions. The sporulation efficiency of the harvested cells was 60% (corresponding to 1.38×10^{11} spores per mL^{-1}).

Table 4. *Altered parameters for batch B2 of B. coagulans fermentation.*

Parameter	Details
C/N ratio	35:1
pH	6.5 ± 0.2 at 0 h, later not controlled
Batch duration	38 h
Mode of operation	Batch

The product of interest in *B. coagulans* fermentation is biomass with enhanced sporulation efficiency. However, concentration of glucose is crucial factor influencing sporulation efficiency in *Bacillus* strains (Young & Spizizen, 1963). Hence, it was planned to reduce C/N ratio for the next batch.

Batch 2. Batch 2 was operated without regulating pH. C/N was reduced to 35:1. This batch was also run as a batch fermentation (Table 4).

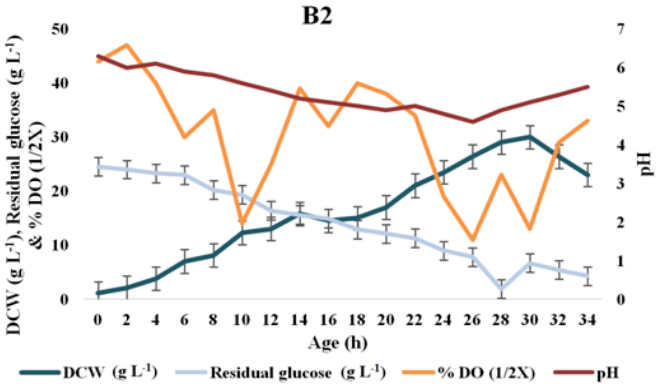

Figure 2. *Fed-batch cultivation profile of batch B2 for B. coagulans at C/N ratio 35:1.*

The results are shown in the Figure 2. Although the duration of the batch was reduced by 6h, higher biomass

yield of 21 g L^{-1} (corresponding to 2.9 × 10^{11} cells per mL^{-1}) was achieved, which was 17% higher than biomass obtained in batch 1. Batch 2 showed sporulation efficiency of 61% (1.7 × 10^{11} gl^{-1} of spores). Thus, sporulation efficiency in batch 2 was similar to the previous batch (60%), but because the biomass yield was higher, total spore count for the cultivation batch 2 as such was higher. Yield of 20 g L^{-1} or more biomass can be termed as HCDF. Thus, the favourable conditions resulting in HCDF for *B. coagulans* were identified.

These results are interesting because there is reduction in glucose requirement (lower C/N ratio of 35:1). Thus the process becomes more economical. One of the ways to improve sporulation efficiency without sacrificing biomass could be to maintain glucose at even lower concentration and make it a fed-batch process. Hence, the third batch was decided to be operated as fed-batch where glucose could be fed intermittently to maintain low carbon source concentration.

Fed-batch 3. This trial was fed-batch fermentation where glucose was added intermittently as shots (Lee et al., 1999). pH was not controlled and the C/N ratio was maintained at 35:1 (Table 5).

Table 5. *Altered parameters for batch B3 of B. coagulans fermentation.*

Parameter	Details
C/N ratio	35:1
Batch duration	30 h
Mode of operation	Fed batch

Trends of residual glucose, pH, DCW (g L^{-1}) and %DO are presented in Figure 3. The culture picked up rapid growth between 4-6 h. % DO trend displayed a sharp drop around 8 h, after which it kept fluctuating till the batch reached stationary phase.

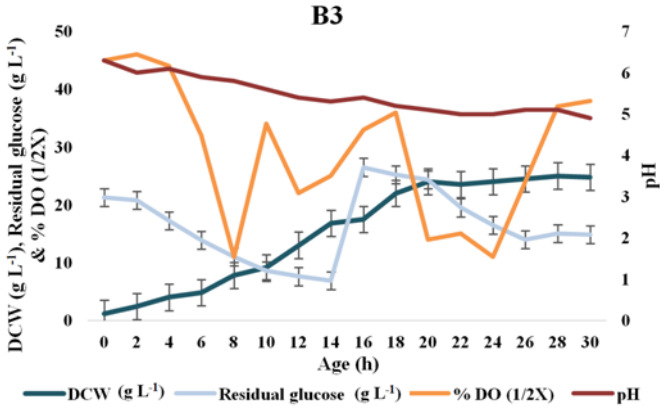

Figure 3. *Fed-batch cultivation profile of batch B3 for B. coagulans at C/N ratio 35:1.*

Batch 3 was harvested at 30 h much earlier than the two previous batches. Yield of 25 g L^{-1} (corresponding to 3.2 × 10^{11} cells per mL^{-1}) which was 20% higher than batch 2, was achieved. In the early stage of cultivation, the cell density was rather low but it picked up later. As the cells grew, the feed rate and oxygen consumption increased and finally the oxygen consumption exceeded the maximum oxygen transfer capacity even when the stirrer speed was at its maximum (600 rpm).

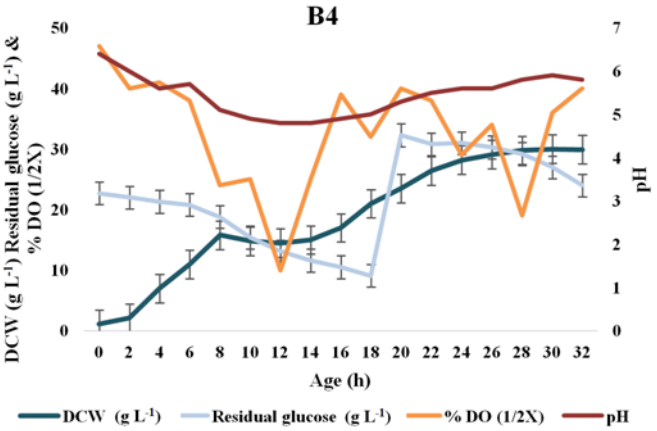

Figure 4. *Fed-batch cultivation profile of batch B4 of B. coagulans at C/N ratio 30:1.*

The pH trend was similar to earlier batches. The glucose present in the starting medium was almost consumed around 16th hour of fermentation. That is when the shot was given (about 50 mL of 250 g L^{-1} glucose concentration), which eventually got consumed in about 28 h of fermentation. The next strategy was to retain the biomass accumulation of 25 g L^{-1} or more and attempt to enhance the spore titres.

Fed-batch 4. Higher C/N ratio might lead to catabolite repression which may prove unfavourable for higher spore production. Hence, C/N was further reduced to 30:1 for batch 4. Trends of residual glucose, pH, DCW (g L^{-1}) and %DO for batch 4 have been presented in Figure 4. This run was a fed-batch with non-regulated pH. Rate of glucose utilization was slow for first 6h of fermentation after which it was consumed rapidly till 18-22 h. Glucose was fed to the batch around the same time (50 mL of 250 g L^{-1} glucose stock). Not very efficient utilization of fed glucose was observed post addition.

The %DO trend appeared to be fluctuating throughout the process, indicating the complex oxygen requirement of cells. However, the sporulation efficiency was highly enhanced (81%). The batch was harvested at the age of 32 h. Batch B4 yielded 30 g L^{-1} of biomass corresponding to 3.8 × 10^{11}cells per mL^{-1} with high spore titre of 1.9 × 10^{11} per mL^{-1}. Thus highly improved productivity was achieved in this batch.

Figure 5 summarizes biomass in terms of cells per mL^{-1} and spore titres (as CFU per mL^{-1} × 10^{-11} after the heat treatment), obtained in the 4 batches. As optimization

progressed batch 4 with C/N ratio of 30:1 and uncontrolled pH, the HCDF yielded the maximum (30 g L^{-1}) of biomass with sporulation efficiency of 81%.

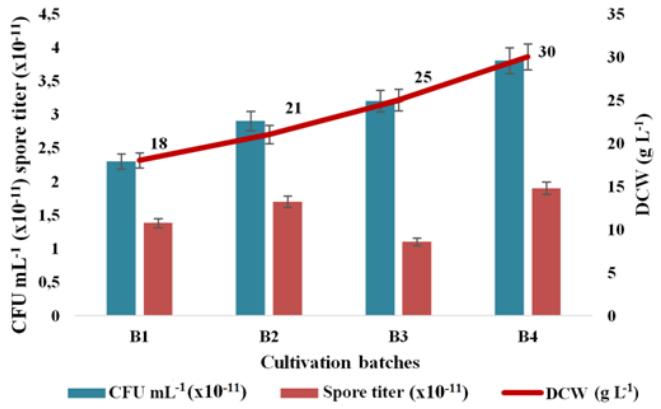

Figure 5. *Summary of biomass and cell titres of the 4 cultivation batches.*

Discussion

The success of cultivation process depends upon the existence of defined environmental conditions for desired product formation. Thus all factors (on-line as well as off-line) which can influence product formation (here biomass accumulation) have to be continuously monitored (Hewitt et al., 2007). The use of fed-batch culture by the fermentation industry takes advantage of the fact that the concentration of the limiting substrate may be maintained at a very low level, thus avoiding the repressive effects of high substrate concentration (in this case glucose). Furthermore, the fed-batch system also gives some control over the organism's growth rate, which is also related to the specific oxygen uptake rate giving some control over the oxygen demand of the cultivation (Stanbury et al., 1997).

Fed-batch fermentation

Shojaosadati et al. (2008), have reviewed the significance of fed-batch cultivation in achieving HCDC. Fed-batch cultivation also eliminates acetate formation due to low levels of glucose in the bioreactor (Hewitt & Nienow, 2007; Shilaoch & Rinas, 2010). It is important that all other nutrients are in excess, so that the growth is solely controlled by the levels of the carbon source present (Riesenberg & Guthke, 1999). This approach allows exponential growth of the culture at a specific rate for much longer duration thereby generating a constant amount of biomass per amount of carbon (Pandey et al., 2000). The right feeding time in fed-batch processes can be followed by monitoring the DO concentration or pH changes. (Mori et al., 1979).

The pH trend

In all the batches, pH initially dropped from 6.5 to 4 and then in the later stages again shifted from 4 to 6-6.5. The trend is in agreement with observations in other fermentations too as in any fermentation pH of an actively growing culture will not remain constant for very long due to their metabolic activities like release of ammonia or CO_2 (Luedeking & Piret, 1959). It was also reported by Gunsalus & Niven, (1942) that normally the pH trend is unidirectional, but in case of certain acid fermentations bidirectional shifts have been observed. All batches displayed a bidirectional pH drift (drop from 6 to 4.5, and again gradual increase to pH 6). The initial drop in pH was likely to be due to conversion of glucose to lactic acid. In the later stage, when almost all the glucose is consumed cells start metabolizing lactate. Hence after 30 h a rise in pH was noted. Reduction in residual glucose concentration with time, indicated the efficient utilization of glucose by the cells.

The DO trend

The %DO of the batches were maintained between 40-60 ± 10%. Irrespective of the different strategies adopted, a steep decline in %DO was indication of initiation of log phase, observed within first 8-10 h of fermentation for all the batches. The %DO then increased gradually and again dropped (Figures 1 to 4). In general, the %DO profiles of all the 4 batches showed fluctuations throughout the fermentation processes. This could be attributed to varying oxygen requirements at various stages of growth of the culture under study. As far as possible the %DO was maintained between 30-70% by altering the agitation rate and/or air flow rate.

The trend obsereved during the optimization batches in line with the reports in the literature. %DO trend of a batch is specific for a culture under a particular set of conditions (Mori et al., 1979; Stanbury et al., 1997; Phue & Shiloach, 2005). As the cells proliferate in a nutritious medium, their count increases and so does their oxygen demand. Hence, the available DO concentration drops, an indication of active growth of cells (log phase). High biomass production can be achieved by satisfying the organism's maximum specific oxygen demand, which is achieved by maintaining the %DO concentration greater than the critical level (20% DO). Oxygen demand of any cultivation largely depends on concentration of the biomass and its respiratory activity, which is related to the growth rate (Mori et al., 1979; El-Mansi et al., 2011).

Foaming and its control

All the 4 batches displayed similar foaming trend. Frothing was observed before inoculation and during first 6 h of fermentation. The foaming observed before inoculation could be attributed to high amino acid and protein content of CSL in the medium. As the cells started multiplying and

metabolizing CSL, frothing reduced and slowly diminished (Rehm & Reed, 1982; Stanbury et al., 1997).

Foam formation can lead to problems like contamination, reduced oxygen transfer, cell lysis and etc. (Ramesh & Lonsane, 1987). The most common cause of foaming are proteins, contributed by medium or due to microbial activities (Lonsane & Karanth, 1988; Stanbury et al., 1997). Silicone (30% v/v) was used as the anti-foaming agent for countering the froth generated during the process. Silicone had no negative effect on the batch productivity.

Sporulation efficiency

The product of interest in probiotic fermentation was biomass. Post-harvest cells are subjected to several harsh processing conditions in the course of formulation development. Maximum sporulation was observed in fed-batch 4 with C/N ratio of 30:1 (81%), while batches 1 and 2 produced biomass with similar sporulation efficiencies (around 60%).

Residual glucose

Several aspects of a fermentation batch can be deduced from utilization pattern of glucose. Examples include: growth rate, stage of growth, rate of biosynthesis and etc. (Stouthamer & Bettenhaussen, 1973).

Biomass formation

DCW estimation showed a gradual increase in biomass from log phase to stationary phase. Careful control, regular and frequent monitoring of the fermentation process for culture *B. coagulans* resulted in an increased biomass production in the initial growth phase due to presence of adequate nitrogen and carbon provided by the medium. Biomass obtained for fed-batch 4 was the maximum (30 g L^{-1}) and least for batch-1 (18 g L^{-1}).

Liu et al. (2000), reported of a fed-batch process for high density cultivation of *Bacillus coagulans* yielding 4.5×10^9 cells per mL^{-1} and spore titre of 1.2×10^9 per mL^{-1}. We here report of a HCDF process which yielded 30 g $L^{-1}$of biomass corresponding to 3.8×10^{11} cells per mL^{-1} and a spore titre of 1.9×10^{11} (an increment of over 100 folds).

Conclusion

A HCDF process was successfully demonstrated on a 6.6 L lab fermentor employing logical and sequential changes in the medium composition and process parameters that resulted in a fed-batch strategy resulting in increase in the productivity of *B. coagulans* fermentation to 30 g L^{-1} DCW and 81% sporulation efficiency. The bioprocess reported here represents 85 times the biomass accumulation relative to previous highest yielding high density cultivation process for *B. coagulans* with spore titres about 158 times their yield. Scale-up of the process from lab fermentor to pilot plant and eventually to a commercial scale can result in substantial cost saving on production scale as the research work described here makes a valuable contribution on HCDF for the probiotic *B. coagulans*.

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Appendix

Trends of %DO, pH, residual sugar and DCW during the fermentation batches

Table 6. *Trends of %DO, pH, residual sugar and DCW during the fermentation batch 1.*

Age (h)	pH	DCW (g L^{-1})	Residual glucose (g L^{-1})	DO%
0	6.30 ± 0.20	1.80 ± 0.50	18.00 ± 3.1	100 ± 3.5
2	65.30 ± 0.30	1.50 ± 1.20	17.80± 2.1	99.3 ± 2.5
4	6.00 ± 0.10	1.80 ± 0.20	16.50 ± 2.0	98.7 ± 3.6
6	6.10 ± 0.15	1.80 ± 1.20	14.80 ± 1.8	83.3 ± 3.0
8	5.90 ± 0.20	2.20 ± 2.20	11.60 ± 3.5	23.2 ± 7.2
10	5.80 ± 0.25	4.30 ± 1.80	10.20 ± 2.3	29.6 ± 6.2
12	5.60 ± 0.21	6.10 ± 0.60	10.00 ± 1.4	13.4 ± 5.2
14	5.40 ± 0.18	8.70 ± 1.20	8.30 ± 1.6	77.8 ± 4.4
16	5.30 ± 0.14	10.80 ± 0.90	7.90 ± 1.8	68.0 ± 5.3
18	5.40 ± 0.20	11.20 ± 0.50	7.20 ± 2.0	79.8 ± 5.3
20	5.20 ± 0.23	12.00 ± 2.60	6.10 ± 2.1	80.0 ± 5.2
22	5.10 ± 0.07	12.00 ± 1.80	6.30 ± 2.3	52.3 ± 8.1
24	5.10 ± 0.20	13.00 ± 2.80	5.80 ± 1.9	47.3 ± 6.2
26	5.00 ± 0.24	17.00 ± 1.60	5.30 ± 1.3	47.9 ± 3.6
28	5.00 ± 0.16	18.00 ± 1.40	5.10 ± 2.2	20.3±7.7
30	5.10 ± 0.11	19.50 ± 1.30	4.90 ± 1.5	47.3 ± 8.2
32	4.90 ± 0.22	20.80 ± 0.30	4.90 ± 0.5	63.3 ± 6.0
34	5.10 ± 0.10	21.00 ± 0.60	5.00 ± 1.6	26.0 ± 7.3
36	4.90 ± 0.23	19.80 ± 1.80	5.00 ± 2.3	130 ± 4.6
38	5.10 ± 0.16	19.20 ± 1.20	4.60 ± 1.0	33.1 ± 7.5
40	5.03 ± 0.11	18.60 ± 2.30	4.10 ± 2.3	47.8 ± 5.2
42	5.80 ± 0.21	18.00 ± 1.80	3.80 ± 2.4	61.6 ± 7.6
44	5.90 ± 0.13	18.00 ± 2.10	3.50 ± 1.5	64.0 ± 11

Table 7. *Trends of %DO, pH, residual sugar and DCW during the fermentation batch 2.*

Age (h)	pH	DCW (g L^{-1})	Residual glucose (g L^{-1})	DO%
0	6.3 ± 0.10	1.1 ± 1.1	24.5 ± 2.0	88 ± 5.3
2	6.0 ± 0.15	2.1 ± 2.3	24.0 ± 1.1	95 ± 2.9
4	6.1 ± 0.12	3.8 ± 1.2	23.3 ± 0.8	81 ± 6.6
6	5.9 ± 0.21	7.0 ± 2.2	23.0 ± 1.4	61 ± 5.2
8	5.8 ± 0.25	8.1 ± 1.1	20.2 ± 1.6	70 ± 6.8
10	5.6 ± 0.18	12.3 ± 0.6	19.3 ± 2.3	29.5 ± 3
12	5.4 ± 0.31	13.0 ± 1.3	16.4 ± 0.7	50 ± 6.3
14	5.2 ± 0.15	15.8 ± 2.3	15.6 ± 1.8	79 ± 3.5
16	5.1 ± 0.12	14.5 ± 0.8	14.9 ± 1.3	65 ±3.8
18	5.0 ± 0.21	15.0 ± 0.7	12.9 ± 0.6	80 ± 2.6
20	4.9 ± 0.20	17.0 ± 1.8	12.1 ± 2.1	76 ± 5.8
22	5.0 ± 0.17	21.0 ± 1.3	11.3 ± 1.5	69 ± 3.5
24	4.8 ± 0.11	23.5 ± 0.9	9.0 ± 2.1	38 ± 5.2
26	4.6 ± 0.21	26.4 ± 1.4	7.8 ± 1.8	23 ± 1.9
28	4.9 ± 0.16	29.0 ± 0.81	1.87 ± 1.7	46 ± 6.5
30	5.1 ± 0.15	30.0 ± 1.1	6.70 ± 0.8	26 ± 4.7
32	5.3 ± 0.23	26.4 ± 0.7	5.40 ± 1.2	59 ± 6.3
34	5.5 ± 0.18	23.0 ± 1.4	4.30 ± 2.3	66 ± 7.2

Table 8. *Trends of %DO, pH, residual sugar and DCW during the fermentation batch 3.*

Age (h)	pH	DCW (g L^{-1})	Residual glucose (g L^{-1})	DO%
0	6.3 ± 0.20	1.2 ± 1.30	21.3 ± 0.20	90 ± 6.30
2	6.0 ± 0.30	2.4 ± 1.50	20.8 ± 1.30	93 ± 7.30
4	6.1 ± 0.12	4.0 ± 2.50	17.2 ± 0.60	88 ± 5.80
6	5.9 ± 0.15	4.8 ± 2.10	13.9 ± 1.80	65 ± 7.80
8	5.8 ± 0.23	7.8 ± 1.90	11.0 ± 1.40	23 ± 4.60
10	5.6 ± 0.09	9.1 ± 0.60	8.6 ± 2.30	68 ± 6.10
12	5.4 ± 0.12	13 ± 1.80	7.6 ± 2.80	45 ± 9.10
14	5.3 ± 1.70	16.8 ± 0.70	6.9 ± 1.20	51 ± 4.20
16	5.4 ± 0.28	17.5 ± 1.90	26.5 ± 1.30	67 ± 3.20
18	5.2± 1.80	22.0 ± 0.60	25.2 ± 1.60	73 ± 5.60
20	5.1 ± 0.05	24.0 ± 2.10	24.3 ± 0.30	28 ± 4.70
22	5.0± 0.12	23.5 ± 0.80	19.5 ± 0.26	31 ± 4.20
24	5.0 ± 0.11	24.0 ± 1.40	16.5 ± 0.14	22 ± 3.50
26	5.1 ± 0.14	24.5 ± 1.90	14.0 ± 0.13	49 ± 8.20
28	5.1 ± 0.16	25.0 ± 1.10	15.0 ± 1.00	74 ± 6.30
30	4.9 ± 0.09	24.8 ± 1.20	14.8 ± 1.10	76 ± 6.80

Table 9. *Trends of %DO, pH, residual sugar and DCW during the fermentation batch 4.*

Age (h)	pH	DCW (g L^{-1})	Residual glucose (g L^{-1})	DO%
0	6.4 ± 0.22	1.10 ± 0.5	22.7 ± 0.5	95 ± 5.5
2	6.0 ± 0.15	2.10 ± 1.4	22.0 ± 0.7	81 ± 6.3
4	5.6 ± 0.13	7.00 ± 0.8	21.3 ± 1.9	82 ± 3.6
6	5.7 ± 0.11	11.0 ± 1.6	20.8 ± 2.5	76 ± 6.1
8	5.1 ± 0.16	15.8 ± 1.6	18.8 ± 1.6	48 ±5.7
10	4.9 ± 0.18	14.8 ± 2.0	15.4 ± 1.3	51 ± 7.8
12	4.8 ± 0.20	14.5 ± 1.7	13.1 ± 0.6	20 ± 3.5

14	4.8 ± 0.14	15.0 ± 2.2	11.6 ± 2.0	50 ± 8.8
16	4.9 ± 0.18	17.0 ± 1.8	10.5 ± 0.7	79 ± 5.7
18	5.0 ± 0.14	21.0 ± 1.2	9.10 ± 1.7	65 ± 7.1
20	5.3 ± 0.15	23.5 ± 1.7	32.3 ± 1.4	80± 4.3
22	5.5±0.20	26.4 ± 1.1	30.8 ± 1.2	76 ± 4.6
24	5.6 ± 0.14	28.2 ± 2.2	31.0 ± 2.3	58 ± 8.3
26	5.6 ± 0.16	29.1 ± 2.6	30.3 ± 1.6	68 ± 4.4
28	5.8 ± 0.60	29.8 ± 1.4	29.2 ± 2.2	38 ± 6.8
30	5.9 ± 0.12	30.0 ± 1.9	27.0 ± 3.1	72 ± 7.2
32	5.8 ± 0.18	29.9 ± 2.1	24.0±1.4	81 ± 4.9