Bioprocess development for enhanced production of probiotic *Bifidobacterium bifidum*

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The objective of this study was the development of bioprocess for enhanced biomass production of probiotic *Bifidobacterium bifidum*. In the first process optimization step in Erlenmeyer flasks cultures, different experiments were conducted to study the effect of inoculum volume, inoculum age, temperature and pH of the growth medium on the kinetics of cell growth. In Erlenmeyer flasks cultures, the maximal biomass production was observed with 1% inoculum of 6 log hours at 37°C, and optimal pH of initial media was found to be 6.0. Further positive development in biomass production was observed by scaling up the fermentation process to stirred tank bioreactor. Fermentation was carried out in 2L stirred tank bioreactor, with agitation of 100 rpm and constant temperature of 37°C. The batch culture produced higher biomass of 34.1 g wet cell weight g/l in 12 log hours and viable counts (2.5×10^9 CFU/ml) compared to Erlenmeyer flasks. In conclusion, batch cultivation in the 2 l bioreactor with this growth medium under optimal conditions gives enhanced biomass production. However, based on our end result, high-cell density fed-batch and pH control strategies are recommended for the commercial production of *B. bifidum* as a probiotic.

Keywords: *Bifidobacterium bifidum*, bioprocess development, culture condition, probiotics.

THE study of gut flora has illustrated the importance of beneficial microorganisms in maintaining good health[1]. Immediately after birth, a complex community of microorganisms conferring a mutualistic beneficial relationship is established in the digestive tract of humans, called the gut microbiome[2]. Intake of broad-spectrum antibiotics to treat bacterial infections and diseases often leads to an imbalance of the gut microbiome. This has been shown to contribute to pathogenesis of diseases such as irritable bowel syndrome (Kennedy, 2014) and inflammatory bowel syndrome[3]. Probiotics are defined by the World Health Organization as 'live microorganisms which, when administered in adequate amounts, confer a health benefit on the host'[4]. To impart a positive therapeutic effect, a minimum of 10^8–10^9 CFU of live probiotics needs be consumed per day[5]. Increasingly, the use of probiotic therapies to restore the gut microbiome and maintain a healthy immune system is being implemented. Controlled intake of probiotic microorganisms confers a myriad of health benefits, including amelioration of symptoms of lactose intolerance, reduction of risk of various other diseases and overall improvement of intestinal health[6]. Probiotics like *Bifidobacterium bifidum* have been characterized for their importance in maintaining and restoring a balanced and healthy gut microbiome. Such beneficial bacteria are being increasingly introduced in the human diet in various forms, including tablet and suspension supplements, and fermented food products to encourage health benefits conferred to the intestinal microflora.

Bifidobacterium is a genus of Gram-positive, non-motile anaerobic bacteria. These bacteria colonize the gastrointestinal (GI) tract within a week following birth, and are one of the dominant commensal bacterial species in the gut of children and adults[7]. *B. bifidum* was first isolated from the faeces of a breast-fed infant[8], and has been extensively studied to reveal clinical benefits[9]. Found naturally in fermented milk products such as yogurt, the last three decades have seen probiotics like *B. bifidum* being increasingly included in a range of commercial and therapeutic products[10].

Large-scale production of bacteria such as *B. bifidum* has gained importance over the past few years in order to cater to the probiotics demand of consumers. Like all microorganisms, the growth characteristics of *B. bifidum* are unique to the organism, and biomass yield during large-scale fermentation is influenced by factors such as medium composition, inoculum conditions (age and percentage), pH and temperature. Growth medium in commercial nutraceutical probiotics production is controlled by nutritional requirement, output requisites and cost of manufacturing[11]. Being an anaerobic bacterium, *B. bifidum* requires a fastidious medium with the right incubation conditions which promote luxuriant growth. *Bifidobacterium* culture requires special medium formulation for optimal growth, since the strains in this genus are not consistent in their nutritional demands[12]. Therapeutic food culture production usually implements a conventional batch fermentation process with suspended cells and optimized conditions from both shake flask and pilot-plant experiments. Although process kinetics factors such as inoculum density and age are pivotal to the productivity and performance of industrial fermentation[13], they are seldom studied or optimized for most probiotic microorganisms.

In the present study, growth characteristics of an inhouse characterized strain of *B. bifidum* have been studied to optimize growth parameter requirements. The *B. bifidum* strain was checked for parameters which may influence biomass, such as inoculums percentage, inoculum age, temperature and effect of medium pH during growth, and has been optimized at shake flask and validated at 2L

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scale fermentor in order to improve the yield in an industrial set-up.

The in-house characterized strain of *B. bifidum* was used in the present study. It was initially streaked on MRS agar[14,15] (BD, France) and incubated at 37° ± 1°C under anaerobic condition, using a BD GasPak (BD, France) for 48 h. Next, 20% glycerol stocks were prepared by anaerobically growing an isolated colony in MRS broth (BD, France) at 37° ± 1°C for 72 h and maintained at –80 freezer[16,17]. All the experiments were carried out using the in-house formulated growth medium comprising dextrose monohydrate 15 g/l, peptone 5 g/l, yeast extracts 10 g/l, $MgSO_4$ 0.050 g/l, $MnSO_4$, 0.020 g/l along with necessary salts (henceforth known as the growth medium).

B. bifidum culture was revived from a glycerol vial in 15 ml growth medium and incubated at 37° ± 1°C. The culture was inoculated in five Erlenmeyer flasks with the growth medium at the following concentrations (v/v): 1.0%, 2.5%, 5.0%, 7.5% and 10.0%. The Erlenmeyer flasks were incubated at 37° ± 1°C for 20 h under anaerobic conditions. At every 4-h interval, samples from each flask were checked for pH, OD_{600} (ref. 18) and wet cell weight (WCW; g/l) (WCW was recorded as g/l at 13,000 rpm for 15 min)[19].

Along with the inoculum volume study, an experiment to determine optimal inoculum age was also set up. *B. bifidum* culture was revived from a glycerol vial in 15 ml growth medium and incubated at 37° ± 1°C for different log hours of growth, namely 6-, 10- and 15-h. At the different log hours, 1% inoculum was transferred to Erlenmeyer flasks containing the growth medium. The flasks were incubated at 37° ± 1°C for 24 h. At every 4-h interval, each tube was checked for pH, OD_{600} and WCW (g/l).

To check the effect of temperature on biomass of *B. bifidum* strain, the following experiment was designed: *B. bifidum* culture was revived from a glycerol vial, and a 1% (v/v) inoculum of sixth log hour was inoculated in five Erlenmeyer flasks containing the growth medium. The flasks were incubated at 30° ± 1°C, 34° ± 1°C, 37° ± 1°C, 38° ± 1°C and 40° ± 1°C for 24 h under anaerobic conditions and at every 4-h interval the samples were checked for pH, OD_{600} and WCW (g/l).

To check effect of initial pH of the growth medium on the growth of *B. bifidum* strain, the following experiment was designed: *B. bifidum* culture was revived from a glycerol vial, and a 1% (v/v) inoculum aged 6 h was inoculated in five Erlenmeyer flasks containing the growth medium. The pH of the growth medium for each of these tubes was adjusted to 3.0, 5.0, 6.0, 7.0 and 9.0 prior to sterilization, using 0.1N sodium hydroxide (NaOH) or 1N orthophosphoric acid (OPA). The tubes were incubated at 37° ± 1°C for 24 h under anaerobic conditions. At every 4-h interval, each tube was checked for pH, OD_{600} and WCW (g/l).

Once the optimum inoculum size, age, pH and temperature were determined, the growth curve of *B. bifidum* was analysed. In order to establish a growth curve for the strain, the culture was revived from a glycerol vial in 15 ml growth medium and incubated at 37° ± 1°C for 24 h. Next, 1% (v/v) seed inoculum (6-h-old) was inoculated to the growth medium. During an anaerobic incubation period of 24 h at 37° ± 1°C, WCW, pH changes and OD_{600} (ref. 18) were recorded at every 2-h interval. WCW was recorded as g/l at 13,000 rpm for 15 min (ref. 19).

B. bifidum biomass production was performed in Erlenmeyer flasks and then validated in a stirred tank bioreactor. Fermentation was carried out in a fully instrumented and computer controlled 2L stirred tank bioreactor (Applikon model Ez-Control bioreactor), equipped with a pH probe and dissolved oxygen (DO) probe. Next, 2 ml of pre-cultured seed was inoculated into 20 ml of the growth medium and cultured for 6 h, which was inoculated into the bioreactor containing 2 l growth medium. Agitation was provided by a standard six-blade impeller at 100 rpm. The pH change during cultivation was monitored using a pH probe attached to a PID controller. The fermentation temperature was maintained using a re-circulating water bath at 37°C, and initial pH was set at a given level. During fermentation, pH and DO were monitored on-line. WCW (g/l) and optical density were monitored offline.

The biomass of the culture during fermentation in the shake flask was calculated by comparing the correlating curves between absorbance values of cell density OD (600 nm) and WCW (g/l). The OD_{600} of the samples was recorded using a spectrophotometer (Jenway Genova Plus, United Kingdom) using with water as the blanking agent and WCW (g/l) was recorded by centrifuging the sample at 8000 rpm for 15 min. The total viable count was determined by pour-plate method. A ten-fold dilution was made by diluting 10 ml of sample with 90 ml of physiological saline. Further, ten-fold serial dilutions, ranging from 10^{-6} to 10^{-8}, were prepared and plated with MRSA or MRS agar medium and the plates were incubated at 37°C for 48 h (refs 20, 21).

Optimization of inoculum percentage was carried out using 12 h inoculum. One per cent, 2.5%, 5.0%, 7.5% and 10.0% of inoculum were transferred to the growth medium. At every 4-h interval, WCW (g/l) and OD_{600} were recorded (Figure 1).

The general WCW (g/l) trend in Figure 1 *a* followed by the samples mimics the growth curve, where an exponential increase in cell mass is observed till the 12th log hour, followed by a stationary phase and then a slight decrease in WCW (g/l) at 24 h (Figure 3 *a*). One percent (v/v) *B. bifidum* seed shows exponential increase in WCW (g/l) till the 16th hour, but seems to decline more rapidly compared to the other inocula. It shows the highest WCW of 31.6 g/l in the growth medium at the 16th

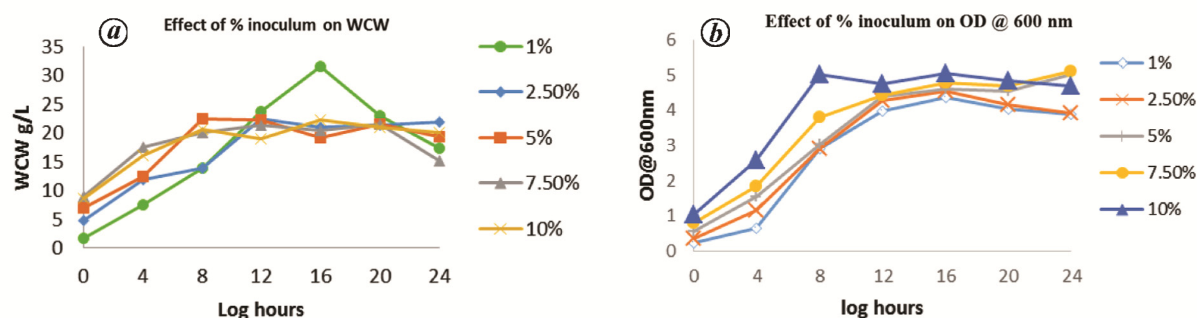

Figure 1. Effect of inoculum volume on the growth of *Bifidobacterium bifidum* at different time intervals. ***a***, Change in wet cell weight (WCW) (g/l); ***b***, OD_{600} value of different of inoculum volumes.

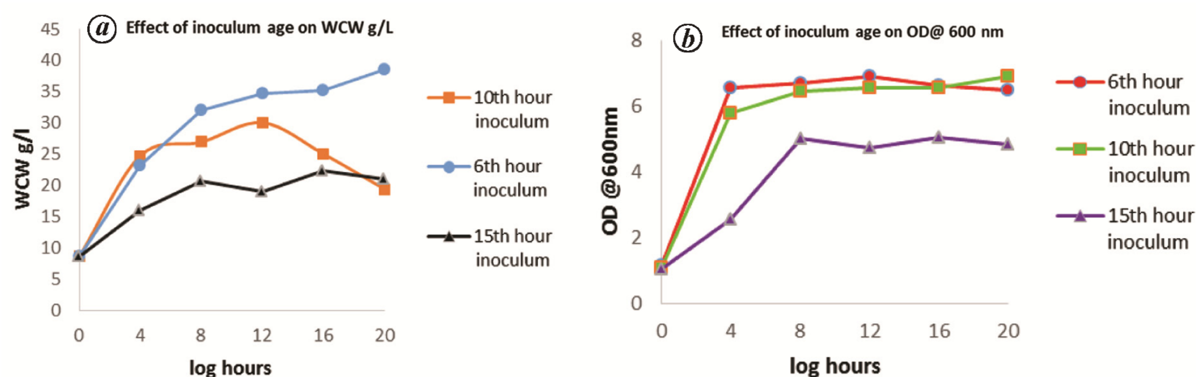

Figure 2. Effect of inoculum age on the growth of *B. bifidum* at different time intervals. ***a***, Change in WCW (g/l); ***b***, OD_{600} value of different inoculum ages.

log hour compared to 2.5%, 5.0%, 7.5% and 10.0% of inoculum.

Figure 1 *b* shows the OD_{600} trend for each of the samples in the study. Once again, the trend observed is comparable to that seen in the growth curve (Figure 5), with all samples showing an exponential rise in OD until the 12th log hour, followed by the stationary phase. As expected, the culture with 10% inoculum shows the greatest rise in OD_{600}, since it also has the highest starting OD_{600} (1.038 compared to 0.219 for 1% inoculum). However, at the 16th log hour, there is no significant difference between the 1% culture and 10% culture OD_{600} values (4.368 and 5.032 respectively). Our finding confirms the use of 1% inoculum in other LAB such as *Lactobacillus acidophilus*[22]. Therefore, the 1% inoculum culture was found to be optimal. Inoculum volume less than 1% was also checked for its efficiency. However, 1% inoculum was found to be optimum (data not shown).

The experimental set-up to determine optimal *B. bifidum* inoculum age included adding a 10% (v/v) inoculum of different log hours, viz. 6-, 10- and 15, to three growth medium-containing flasks. At every 4-h interval, WCW (g/l) and OD_{600} were recorded for 20 h of growth (Figure 2).

The general trend observed in Figure 2 *a* for all ages of inoculum is a steady increase in WCW (g/l) until 12 h of growth, followed by either a stationary phase or a slight decline in growth between 16 and 20 h. Those data are consistent with growth of *B. bifidum* and WCW (g/l) data from previous experiments. The 10-h inoculum shows a relatively steep decline in WCW (g/l) between 12 and 20 h, with a 1.4-fold decrease in WCW (g/l) at the 20th log hour compared to the 12th log hour. The WCW (g/l) of the 6-h inoculum does not decline over the growth phase. The 15-h inoculum, while following the aforementioned WCW (g/l) trend, shows an overall lower WCW (g/l) compared to the 6- and 10-h samples (19 g l^{-1} at 12th log hour, compared to 32 and 27 g l^{-1} for 6- and 10-h samples respectively).

In Figure 2 *b*, the effect of different inoculum ages on OD_{600} and growth of *B. bifidum* can be observed. The general trend followed is an exponential increase in OD_{600} within 8 log hours of growth, which is maintained till 20 log hours with negligible changes. These data are consistent with inoculum volume results data obtained. Amongst the three samples, the 6 h inoculum shows greatest rise in OD_{600} (5.66-fold rise between 0 and 4 log hours, compared to 5.30 and 2.47 for 10-h and 15-h inoculum respectively), as well as highest OD at 12th log hour of growth (6.888). The 10-h inoculum grows comparably with the 6-h inoculum, with a slightly lower OD at the fourth log hour of growth (5.792 compared to 6.544

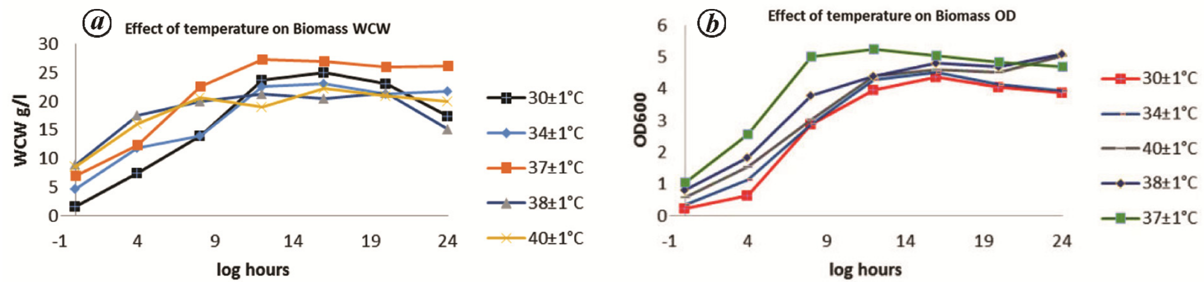

Figure 3. Effect of temperature on *B. bifidum* growth at different time intervals. ***a***, Change in WCW (g/l); ***b***, OD_{600} values for the different temperatures.

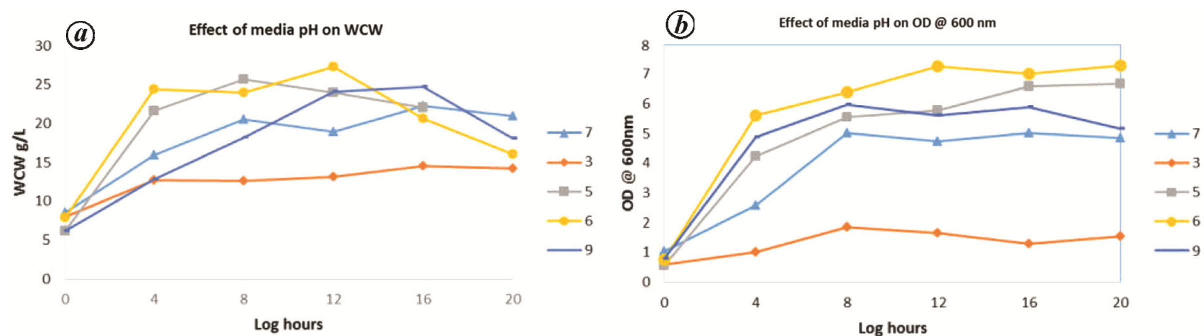

Figure 4. Influence of initial media pH on *B. bifidum* growth at different time intervals. ***a***, Change in WCW (g/l); ***b***, OD_{600} values of different pH of the initial medium.

for the 6-h sample). On the other hand, the 15-h inoculum shows a significantly lower OD_{600} rise and change compared to the other two samples as the culture grows over 20 h. The maximum OD achieved by this sample is seen at 8 log hours, and is 5.024 compared to 6.704 and 6.456 of the 6-h and 10-h inoculum respectively. On the basis of the growth curve established above, at the sixth log hour, our strain of interest is in log phase of growth and is undergoing active cell division. This presents itself as a suitable explanation for the 2.5-fold increase in OD_{600} between the 6-h and 15-h (stationary phase) inoculum in the sixth log hour of growth. While the 10-h inoculum also shows a similar OD_{600} profile as the 6-h inoculum, both time and bioreactor running cost can be reduced upon using the 6-h inoculum, to yield similar *B. bifidum* biomass. Inoculum age less than 6-h was also checked for efficiency; however 6 h inoculum was found to be optimum (data not shown).

To check the optimum temperature for maximum biomass production of *B. bifidum*, five flasks were inoculated with seed and incubated at different temperatures 30 ± 1°C, 34 ± 1°C, 37 ± 1°C, 38 ± 1°C and 40 ± 1°C for 24 h under anaerobic conditions. At 4-h intervals, OD_{600} and WCW (g/l) were recorded for 20 h of growth (Figure 3). The WCW and OD_{600} trend for different temperatures can be observed. In this study, WCW at 37 ± 1°C at 12 log hours was observed to be more optimal at 27.3 g/l with corresponding OD_{600} of 5.251. Ram and Chander[23] reported the optimum temperature to be 37°C for *B. bifidum* and found that the culture grown at 37°C shows higher percentage adhesion in the human intestine; this may be because of higher increased synthesis of hydrophobins at 37°C.

To elucidate whether the initial pH of the medium has an effect on growth of the strain of interest, five media of different pH values were designed: 3.0, 5.0, 6.0, 7.0 and 9.0, and 1% of a 6-h inoculum was added. At 4-h intervals, OD_{600} and WCW (g/l) were recorded for 20 h of growth (Figure 4). Growth of *B. bifidum* was pH-dependent. In Figure 4 *a*, the WCW (g/l) trend for different initial media pH values of the media can be observed. The *B. bifidum* formulated growth medium has an initial pH of 6.9. In this study, the WCW changes in the pH 7 sample are seen to be in conjunction with the growth curve pH changes (Figure 5). The growth of pH 3 sample is comparatively significantly lower to all other samples (13.2 g l^{-1} at 12th log hour compared to the others, which are in the range 19.0–27.3 g l^{-1}). Growth of the pH 5.0 and pH 6 samples, on the other hand, is comparatively higher than the other samples, with highest WCW of 25.7 and 27.3 g l^{-1} respectively, observed at 12 log hours of growth.

The overall OD_{600} trend mimics the above studies, with the pH 7 sample showing most similarities (Figure 4 *b*).

As seen with the WCW (g/l) data, pH 3 sample shows least rise in OD_{600} over the growth phase, with a maximum of 1.850 at eighth log hour, and an average rise of 0.316 h^{-1} between zeroth and eighth log hour. This is much lower when compared to pH 5 and pH 6 samples (1.250 and 1.407 h^{-1} respectively). Furthermore, when compared to pH 7, once again, the samples with initial medium pH of 5 and 6 both show higher OD throughout growth. Our findings in this regard are consistent with the observations of Mlobeli[24], who reported that optimal pH for *B. bifidum* is between 4.9 and 6.5. It was reported by Known *et al.*[25] that the production ratio of A : L (acetic acid to lactic acid) is pH dependent and it will vary with changes in pH and pH 6.0 as optimum for *B. bifidum* in submerged membrane bioreactor fermentation.

The *B. bifidum* strain was anaerobically grown for 24 h in the growth medium, with pH, WCW (g/l) and OD_{600} measured every 2 h. Figure 1 shows this growth curve as elucidated by OD_{600}, WCW (g/l) and pH.

The OD_{600} profile of *B. bifidum* shows an exponential increase till the tenth log hour, starting at 1.040 and reaching up till 5.24, and plateaus for the next 14 h. The growth curve of *B. bifidum* ascertained by OD_{600} is correlated with the WCW (g/l) data. An increase in the WCW (g/l) is observed till the eighth log hour; which later slows down between 10 and 22 log hours. The pH of the culture medium decreases from 7.2 to 4.7 immediately after addition of inoculum, and stays between 4.0 and 4.4 throughout the growth phase of the culture.

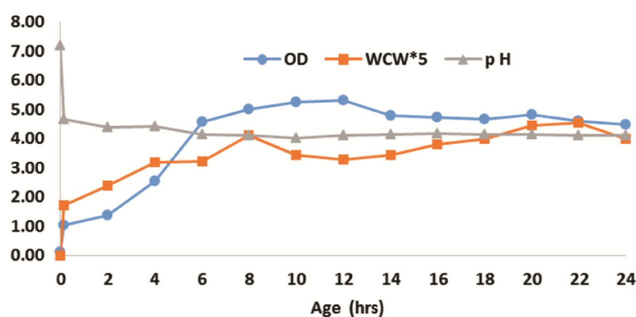

Figure 5. *B. bifidum* growth curve. This graph illustrates the OD, WCW (g/l) and pH trends followed by this strain over 24 h of growth.

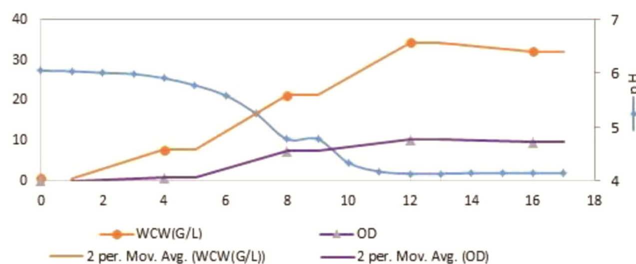

Figure 6. Batch trend in 2L fermentor (Applikon model Ez-Control bioreactor). Graph illustrates OD, WCW (g/l) and pH trends (uncontrolled) in batch fermentation at 2L scale.

Once the most important variables and their optimum values were obtained, validation in the bioreactor was performed in order to confirm the optimized culture conditions. Mechanical stirring to produce *B. bifidum* biomass in the bioreactor allows greater homogenization of the cells in contact with the medium, thus increasing nutrients transfer and biomass. Biomass production of *B. bifidum* improved significantly in the optimized growth medium and in the bioreactor compared with Erlenmeyer flasks. The highest biomass 34.1 g/l (WCW) was obtained at the 12th log hour and pH in batch fermentation started decreasing from 6.04 (initial medium) to 4.14 at 14 log hours and become constant till 17 log hours. Once the WCW and pH became constant (17 log hours), the batch-harvested and end of fermentor broth was analysed for total viable count by ten-fold serial dilution. The total viable count was found to be 2.5×10^9 CFU/ml.

This study aimed to identify and optimize the growth environment and requirements of an in-house characterized *B. bifidum* strain in both shake flask and small bioreactor. The optimum values obtained after the study were pH 6, inoculum volume 1% (v/v) , inoculum age 6 h at 37°C resulting in the maximum yield of probiotic bacteria. The nutraceutical and therapeutic microbiology industries are constantly ascertaining, authenticating and troubleshooting experimental protocols to enable successful large-scale production of viable and safe probiotics. Further prospects for this strain include characterizing its behaviour in a large bioreactor, and fed-batch fermentation could further increase biomass yield or reduce fermentor costs.

Compliance with ethical standards: This communication does not contain any studies with human or animal subjects.

Conflict of interest: The authors declare that they have no conflict of interest.

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Pigment analysis of palm leaf manuscripts of India

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The primary aim of this study is to scientifically examine major pigments and binders used in Indian palm leaf manuscripts. The colours in four painted palm leaf manuscripts (18th–19th century CE) were observed using a digital microscope and SEM-EDX. Analytical studies showed that the colours were prepared using traditional Indian techniques by mixing primary pigments like black, red, white, yellow, blue and green with locally available gum or adhesive. The prominent appearance of black or brown stains, discolouration and insect activity were the major causes for making the leaves brittle and unstable. The identification of pigments also provided rich source of information about the composition and development of painting traditions on palm leaf in India`s southern and eastern parts. Laboratory studies also revealed the use of mineral as colourant for Indian palm leaf illustrations.

Keywords: Binders, palm leaf manuscripts, painting traditions, pigments.

THE earliest imprints of human activities in India have always been through oral tradition. As part of Hindu tradition, knowledge has been transmitted orally to the future generations. The emphasis of spoken language led to the negligence of writing tradition. India was always considered a land of tradition which has transmitted orally from one generation to another until the writing system came into existence. The concept of writing system in India started much later than its language. The first reference to writing in India was found in Pali Buddhists canon of the 5th century BC (ref. 1). Consequently, writing became a means of transmitting and documenting knowledge that would otherwise be lost[2]. Hence the recorded knowledge in the form of manuscripts has played an essential role in the development, preservation and propagation of learning and literature in India, down the ages. Recording them in writing form on a large scale was the only solution to preserve and propagate them for future generations.

India is a hub of the oldest traditions in the form of manuscripts. Before the invention of paper, various mediums such as stone, clay tablet, metal, wood, birch bark

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