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A Comprehensive Bioprocessing Approach to Foster Cheese Whey Valorization: On-Site β-Galactosidase Secretion for Lactose Hydrolysis and Sequential Bacterial Cellulose Production

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Abstract: Cheese whey (CW) constitutes a dairy industry by-product, with considerable polluting impact, related mostly with lactose. Numerous bioprocessing approaches have been suggested for lactose utilization, however, full exploitation is hindered by strain specificity for lactose consumption, entailing a confined range of end-products. Thus, we developed a CW valorization process generating high added-value products (crude enzymes, nutrient supplements, biopolymers). First, the ability of *Aspergillus awamori* to secrete β-galactosidase was studied under several conditions during solid-state fermentation (SSF). Maximum enzyme activity (148 U/g) was obtained at 70% initial moisture content after three days. Crude enzymatic extracts were further implemented to hydrolyze CW lactose, assessing the effect of hydrolysis time, temperature and initial enzymatic activity. Complete lactose hydrolysis was obtained after 36 h, using 15 U/mL initial enzymatic activity. Subsequently, submerged fermentations were performed with the produced hydrolysates as onset feedstocks to produce bacterial cellulose (5.6–7 g/L). Our findings indicate a novel approach to valorize CW via the production of crude enzymes and lactose hydrolysis, aiming to unfold the output potential of intermediate product formation and end-product applications. Likewise, this study generated a bio-based material to be further introduced in novel food formulations, elaborating and conforming with the basic pillars of circular economy.

Keywords: cheese whey; *Aspergillus awamori*; β-galactosidase; lactose hydrolysis; *Acetobacter xylinum*; bacterial cellulose

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1. Introduction

Agro-industrial waste and by-products streams occur in each step of the food supply chain, specifically during processing. These streams, however, still contain compounds of importance to develop further exploitation schemes, considering also the transition from a linear to circular bioeconomy. Likewise, cheese whey (CW) corresponds to an unavoidable by-product stream of the dairy industry, receiving critical attention because of the high environmental burden, but also owing to the several components with beneficial nutritional and functional properties [1,2]. The compositional analysis of the onset material usually outlines the deployment of subsequent valorization routes within a biorefinery concept to generate high added-value products along with zero waste. For instance, up to date, the vast majority of studies related to the utilization of CW through bioconversion processes implement the application of microbial entities able to consume lactose [3–6]. As a result, the range of end-applications, particularly sustainable food production, is restricted. Alternatively, whey lactose fraction could be hydrolyzed to the respective monosaccharides and

further studied in fermentation processes. Apart from the conventional chemical methods for lactose hydrolysis, previous studies have also undertaken enzymatic hydrolysis [7,8].

Lactose hydrolysis is accomplished via the action of galactosidases, which are ubiquitous enzymes with complex structures. Galactosidases confer several advantages in food industry, including the manufacture of lactose-free dairy products or galacto-oligosaccharides synthesis through transglycosylation reactions [8,9]. Bacterial, yeast and fungal strains correspond to microbial sources of β-galactosidase (β-gal; EC 3.2.1.23 commonly known as lactase), attracting significant interest owing to the ability to secrete the enzymes extracellularly along with featuring properties such as high catalytic activity and reaction rate [10]. On top of that, environmentally benign enzyme production using crude renewable resources as low-cost media has been demonstrated by several species [11]. Notably, several *Aspergillus* species constitute key producers for sustainable and cost-effective enzymes production, also classified as "generally recognized as safe" (GRAS) by the Food and Drug Administration [12]. Currently, evidence for β-galactosidase production exists in the closely related strains of *Aspergillus lacticoferratus* and *Aspergillus awamori* [13,14]. In particular, *A. awamori* produces various hydrolytic enzymes such as glucoamylase, protease, phytase, β-glucosidase, β-xylosidase and cellulases useful for agro-industrial by-product-stream valorization [15–18].

The development of effective and feasible consolidated biorefining should include raw materials with consistent composition, yearlong supply and engage the holistic exploitation of each valuable compound for further novel applications. Extensive studies have been performed to utilize CW derived lactose for the fermentative production of several microbial metabolites [19]. Equally, the protein fraction prevailed in studies targeting novel food formulations [19]. However, the ideal concept would encompass the valorization of both protein and lactose fractions within the same biorefinery approach. Likewise, targeted intermediate products (e.g., biodegradable polymers) within a biorefinery process could be used as onset materials to elaborate "de novo" diversified novel formulations. Bacterial cellulose (BC) is a natural extracellular polysaccharide demonstrating prominent food and biomedical applications, also characterized as GRAS dietary fiber by the FDA in 1992 [20]. Numerous research studies have suggested the use of BC in food applications, including as a flavor additive, fat replacer, stabilizer, rheology modifier and meat analog [20]. Few recent studies also indicated the use of BC as an edible carrier for cell cultures, enzymes, antimicrobial compounds or even biocolorants [21–23]. Despite the simple downstream processing steps, industrial BC production is hindered owing to the high cost of conventional synthetic media. Therefore, agro-industrial by-products and food waste streams have been previously assessed as fermentation supplements for cost-effective BC production [24–27].

Our ultimate target is to develop a holistic approach to exploit cheese whey fractions to generate value-added products, with potential food formulations. Likewise, this initial study describes a two-stage bioprocess to produce crude β-galactosidase and proteases using *A. awamori*, followed by enzymatic hydrolysis of whey lactose, to formulate a nutrient rich feedstock. BC was selected as a case study of an intermediate value-added product. The optimization of crude enzymes production and enzymatic hydrolysis was undertaken via the assessment of several crucial parameters that affect enzyme secretion (e.g., pH value, temperature, enzyme loading). The performance of enzymatic hydrolysis was also assessed, and the obtained hydrolysate was subsequently evaluated as a crude nutrient supplement to generate BC.

2. Materials and Methods

2.1. Microbial Strains and Media

A. awamori strain 2B.361U 2/1 was kindly provided by Dr Apostolis Koutinas (Agricultural University of Athens, Athens, Greece) and was employed for the generation of crude enzymes and cheese whey hydrolysis. Fungal strain origin and revival protocols have been reported in a previous publication [16]. Microorganisms were sub-cultured and

stored at 4 °C in agar slopes containing 5% (*w*/*v*) wheat bran (WB) and 2% agar (*w*/*v*). For inoculum preparation, the fungus was grown for 5 days at 30 °C on identical solid substrate to sporulate. *A. xylinum* strain 15,973 purchased from DSMZ culture collection was used for bacterial cellulose (BC) production. Bacterial stock was preserved at −80 °C. For BC production, inocula preparation was performed on Hestrin–Schramm's medium (HS) [27]. The microorganism sub-cultures were grown at 30 °C for 48 h under agitation (180 rpm) [28]. Wheat bran that consisted of 26% (*w*/*w*) carbohydrates, 14% (*w*/*w*) proteins and 0.01% (*w*/*w*) salt, was purchased from a local market. Deproteinized (after "myzithra" cheese manufacturing) cheese whey (approximately 50 g/L lactose) was kindly provided by "Galiatsatos" dairy company (Kefalonia, Greece).

2.2. Crude Enzyme Production and Cheese Whey Hydrolysis

Crude enzyme production was determined during solid state fermentations (SSF) on wheat bran (WB) and further optimized under various parameters. More specifically, 5 g WB (dry basis) were weighed and sterilized into 250 mL Erlenmeyer flasks. To enhance secretion of fungal β-galactosidase, the medium was supplemented with 10 mg $MgSO_4$ in each SSF culture [13,29]. Suspensions of approximately 2×10^6 spores mL^{-1} were prepared by collecting spores of 5 days old fungal pre-cultures as described above. Inoculated WB flasks were incubated at 28 °C under static conditions and enzyme activity was determined at regular time intervals until 120 h of incubation. In terms of enzyme production optimization, different initial moisture content of the substrate of 60, 65, 70 and 75% (*w*/*w* on a dry basis) was also examined. The varying moisture content was fixed by addition of deproteinized whey (pH 4.5) in order to stimulate enzyme production.

At the end of the fermentation process, the WB solids were mixed thoroughly with deproteinized whey (1:10 *w*/*v*) at 120 rpm for 1 h at room temperature [30]. Crude enzyme extracts were filtered through sterile gauze and centrifuged further at 4000 rpm for 20 min. The effect of temperature in the hydrolytic activity of the enzymes was evaluated at 40–70 °C for 60 h. Lactose hydrolysis assay was further optimized employing varying initial enzyme activities of 7.5, 11 and 15 U/mL and hydrolysis experiments were carried out at 500 mL final volume in a water bath for 60 h under agitation. Initial enzyme activities used in hydrolysis experiments were achieved by selecting the appropriate amount of crude enzymes (~150 U/g), which were produced under optimal SSF conditions. Samples for sugars and free amino nitrogen (FAN) determination were collected at regular time intervals and heated (100 °C) to inactivate enzymatic reaction. Subsequently, the pH value of hydrolysates was adjusted to 6.0, and they were sterilized to be used as nutrient supplements for BC production. All the experiments were performed in duplicates.

2.3. Submerged Fermentation and Bacterial Cellulose (BC) Production

Cheese whey was pretreated with crude β-galactosidase extracts to break down lactose, and the produced hydrolysates were further evaluated for the production of BC by *A. xylinum*. In addition to that, experiments with unhydrolyzed CW, including initial CW of 50 g/L (A), CW diluted to 25 g/L (B) and CW diluted and supplemented with yeast extract (C), were also performed for comparative reasons. Experiments were conducted in 250 mL Erlenmeyer flasks containing 50 mL of hydrolysate (pH 6.0). The substrate was inoculated with 10% (*v*/*v*) of 48 h bacterial sub-cultures and incubated at 30 °C on a 10 days static cultivation. Sugars along with FAN consumption were determined during fermentation. The obtained BC was pretreated as described by Żywicka et al. [31] with slight modifications. Briefly, samples were purified with 0.1% NaOH at 80 °C for 30 min to inactivate the bacterial cells and remove medium components. BC membranes were washed in distilled water until the pH stabilized. Further on, the membranes were air-dried at 40 °C until constant weight and stored at room temperature for future use [32]. All the experiments were performed in duplicates.

2.4. Analytical Methods

Sugar concentration during CW hydrolysis and fermentation process were quantified by high performance liquid chromatography (HPLC) analysis (1200 series Agilent, Santa Clara, CA, USA) equipped with a differential refraction detector and an Aminex HPX-87H column (300 mm length × 7.8 mm internal diameter). The mobile phase was 10 mM H_2SO_4. The analysis was performed under isocratic conditions at a flow rate of 0.6 mL/min and 65 °C column temperature [33]. Injection volume was 10 μL and run time for samples was 25 min. Before injection, samples were diluted to appropriate concentration and filtered through a 0.22 μm Whatman® (Maidstone, UK) membrane filter.

Protease activity was evaluated by the production of free amino nitrogen (FAN) after hydrolysis of 7.5 g L^{-1} of casein in 0.2 M phosphate buffer (pH 6.0) at 55 °C for 30 min. One unit (U) of proteolytic activity was defined as the amount of enzyme required for the release of 1 μg FAN in one minute under the above conditions [34]. FAN concentration was determined in both hydrolysis and fermentation using the ninhydrin colorimetric method [35].

Production of β-galactosidase was measured by the o-nitrophenol-β-d-galactopyranoside (ONPG) assay according to Raol et al. [29] with slight modifications. Briefly, 0.1 mL of crude extract was added to 0.4 mL of ONPG (3.0 mM) dissolved in sodium citrate buffer (50 mM, pH 5.0) and incubated at 50 °C for 10 min. The reaction was terminated by the addition Na_2CO_3 (0.1 M) and the release of o-nitrophenol was estimated spectrophotometrically at 420 nm at a final volume of 3.0 mL. A calibration curve was prepared with o-nitrophenol under the same conditions. One unit (U) of β-galactosidase was defined as the amount of enzyme catalyzing the release of 1 μmol of o-nitrophenol per min according to the absorbance measurement.

2.5. Statistical Analysis

Results are presented as mean values ± standard deviation. Statistical analysis was performed by applying analysis of variance (ANOVA) to evaluate the variations between group means (between treatment effect). Tukey HSD post-hoc test with 95% confidence intervals was used to indicate significant differences between hydrolysis levels and bacterial cellulose production.

3. Results and Discussion

3.1. Solid State Fermentation (SSF) and Crude β-Galactosidase Production

The leading target of this study was to evaluate the hydrolytic activity of *A. awamori* on CW to obtain a nutrient-rich supplement deriving from lactose hydrolysis, that will substitute synthetic media in a following bioconversion process. Therefore, SSF optimization to enhance β-galactosidase production using WB as a single substrate was initially undertaken, based also on previous studies that have outlined that WB reinforced β-galactosidase production [36]. This has been attributed to the appropriate ratio of hemicellulose to sugars, that is defined as a stimulus factor for galactosidase production [37]. Figures 1 and 2 demonstrate the effect of initial moisture content, ranging from 60 to 75%, along with incubation time (1–5 days). Maximum production of β-galactosidase reached 148 U/g (db) at 70% of initial moisture after 70 h of fermentation. Earlier reports highlighted that increased moisture levels enhanced β-galactosidase yield in *A. tubingensis* [29]. The latter usually associates with the fact that moisture crucially affects nutrient solubility within the substrate [38]. As it can be easily observed, the production rate exhibits an increasing trend (Figure 1), during the first three days of fermentation followed by a decrease after approximately 70 h (three days) of incubation. Similar results were also obtained in studies using *A. tubigensis* and *A. awamori*, respectively [14,29], whereby prolonged fermentation times entailed higher β-galactosidase activities. For instance, Nizamuddin et al. [30] demonstrated optimum β-galactosidase production by *A. oryzae* after seven days of incubation, Raol et al. [29] found maximum enzyme activity by *A. tubingensis* at seven

days, whereas Cardoso et al. [13] performed SSF for six days to produce β-galactosidase production by *A. lacticoffeatus*.

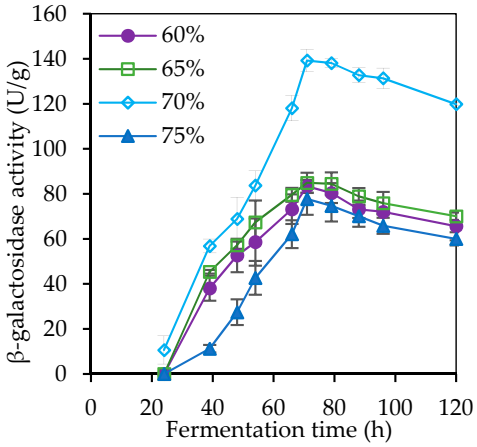


Figure 1. Effect of solid state fermentation (SSF) time in crude β-galactosidase production by *A. awamori*, at different initial moisture contents.

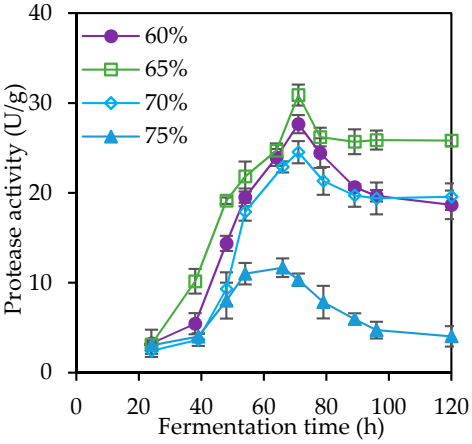


Figure 2. Effect of solid state fermentation (SSF) time in crude protease production by *A. awamori*, at different initial moisture contents.

Recently, Vidya et al. [14] studied α- and β-galactosidase production from *A. awamori* (MTCC 548), whereby the purified enzyme exhibited 25.5–176.5 U/mg of activity, respectively. On top of that, the authors reported β-xylosidase and β-glucosidase activities, suggesting the ample substrate specificity. Several preceding studies had also suggested multi-enzyme production by *A. awamori* including glucoamylase and protease [16,39]. Therefore, proteolytic activity was also undertaken (Figure 2), reaching the highest value after 70 h of fermentation (30.9 U/g). Similarly, Wang et al. [40] reported protease activities up to 40 U/g, (db) after 120 h employing similar SSF conditions. Evidently, it could be speculated that the addition of CW in SSF cultures, induced the secretion of β-galactosidases considering that fungal strains tend to adapt in the environmental niches and develop mechanisms for the production of specific enzymes. Moreover, this could be attributed to the low pH during fermentation, that could potentially enhance *Aspergillus* β-galactosidase production [11,13,41] Ultimately, SSF time for crude β-galactosidase and protease was standardized at 70 h to obtain maximal activities, that would be implemented in subsequent hydrolytic reactions of CW.

3.2. Cheese Whey Hydrolysis Study

CW hydrolysis was performed using the crude enzymatic extracts obtained from SSF cultures. Figure 3a illustrates the results obtained from different hydrolysis temperatures,

whereby it can be observed that 60–65 °C was the optimum hydrolysis temperature of *A. awamori*. Ultimately, at the end of the bioprocess, crude enzymes hydrolyzed >90% of the initial whey lactose (Figure 3a). On the other hand, the optimum proteolytic activity was observed at 55 °C, as it has been earlier indicated by Tsakona et al. [16]. More particularly, as displayed in Figure 3b, FAN production increased along with the increase in temperature up to 55 °C, followed by a gradual reduction with further temperature increments (Figure 3b). Based on our results, significant differences ($p < 0.05$) were observed on the performed hydrolyses, at almost all evaluated temperatures. Likewise, no significant differences ($p > 0.05$) were observed on hydrolysis experiments carried out at 60 and 65 °C. Previous studies have also demonstrated processing of cheese whey via the implementation of microbial β-galactosidase to generate value-added products [42,43]. Generally, temperatures ranging between 50 and 60 °C and acidic pH values (3.5–4.5) have been reported as the optimal conditions for fungal β-galactosidase activity [13]. Additionally, Silvério et al. [44] recently studied β-galactosidase production in several *Aspergillus* species, aiming to synthesize potential prebiotics, whereby an increased enzyme activity in the range of 50–60 °C was noted. The current observation highlights the significant potential of the enzymes, since thermal stability is of imperative practical use for diverse bioprocesses, preventing various contaminations [45,46]. Furthermore, the results obtained postulate that the enzyme is more accessible during the first hours of hydrolysis. More specifically, a higher hydrolysis rate during the first 12 h entailed 30–50% of lactose hydrolysis, followed by a decreased rate at prolonged incubation time. Several studies also coincide with such findings where hydrolysis products decreased or even restricted lactose hydrolysis reaction [41,47,48]. Indeed, it has been previously established that at high galactose concentrations, β-galactosidase activity is impaired since the conformational modification of the enzyme's active site reduces the affinity for its substrate [49,50]. Moreover, galactose could also act as a competitive inhibitor of β-galactosidase via the formation of galactosyl–enzyme intermediate products [51].

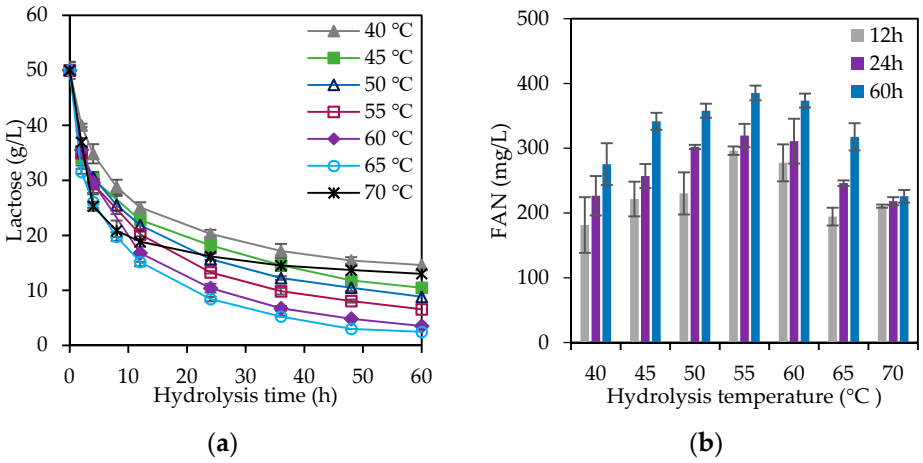


Figure 3. Effect of temperature on cheese whey (CW) hydrolysis using crude β-galactosidase and proteases. Kinetics of (**a**) lactose hydrolysis and (**b**) free amino nitrogen (FAN) production.

In an effort to further optimize whey hydrolysis, trials were also performed to evaluate the effect of different initial enzymatic activities on lactose breakdown and FAN production. Initial enzymatic activities of 7.5, 11, 15 U/mL were employed, and the results are illustrated in Figure 4. Figure 4a presents the kinetic profile of lactose hydrolysis, whereas Figure 4b presents FAN production in specific timepoints. Evidently, the use of 15 U/mL resulted in accelerated rates and complete lactose hydrolysis at 36 h and the production of 583.13 mg/L FAN. On the other hand, initial enzymatic activities of 7.5 and 11 U/mL yielded 87 and 93% of hydrolysis, respectively, at the same time point, providing lower productivities. Even though the degree of hydrolysis seems to follow a dose-dependent trend, apparently much higher concentrations do not significantly alter the hydrolysis efficiency, although

complete hydrolysis is performed significantly earlier at higher initial enzymatic activities. Worth noting, FAN production increased almost two-fold at higher initial enzymatic activities. Rosolen et al. [52] also presented similar efficiency levels on whey lactose hydrolysis by *A. oryzae*, regardless of the enzyme concentrations used (3, 6 and 9 U/mL). This observation probably also indicates the saturation of lactose at high β-galactosidase concentrations [53]. Thus, as in previous studies, our results imply that CW lactose hydrolysis is not strictly proportional with enzyme concentration [7,54]. However, complete hydrolysis was performed in almost half the time, using 15 U/mL, compared with the case of 7.5 U/mL. Nonetheless, in the event that scale up should be considered, lactose hydrolysis efficiency and FAN production should coincide with the feasibility of the process to highlight the most favorable operating conditions, which will be designated by the end target products.

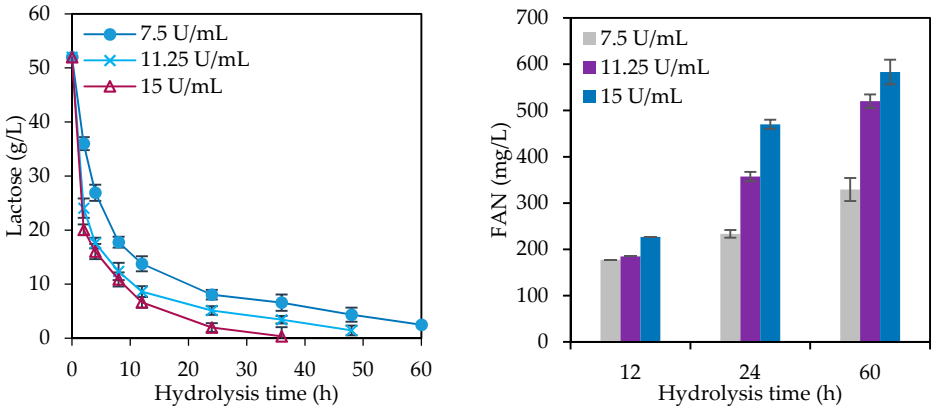
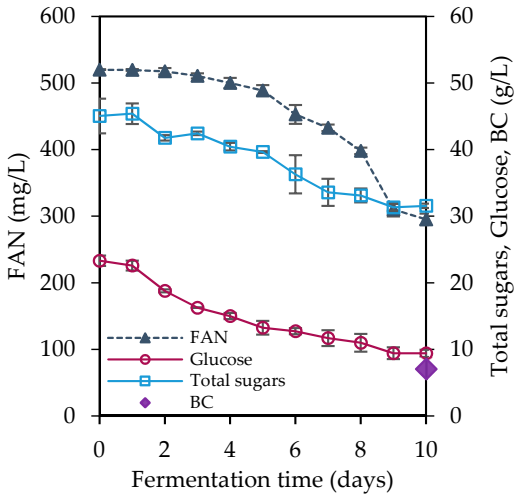


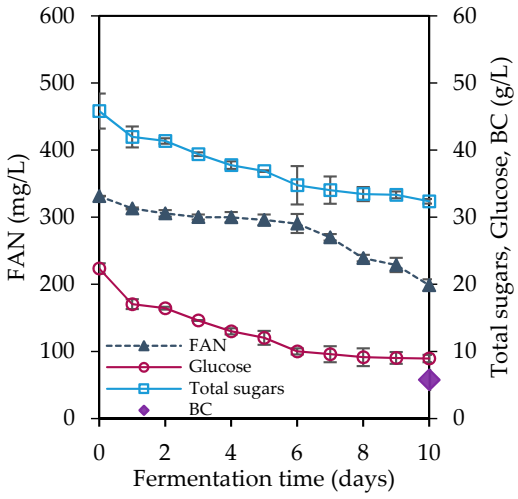
Figure 4. Effect of different initial enzymatic activity of crude β-galactosidase on cheese whey (CW) hydrolysis. Kinetics of (**a**) lactose hydrolysis and (**b**) free amino nitrogen (FAN) production.

3.3. Bacterial Cellulose Production

CW constitutes a renewable, zero-cost substrate suitable for microbial fermentation, mostly requiring minimal pretreatment. However, often lactose does not undergo fermentation by several microorganisms including acetic acid bacteria. Previous reports demonstrated low BC-production from unhydrolyzed CW, thus hindering further implementation. Thus, pretreatment is often essential to overcome such limitations. Besides this, only limited studies have evaluated CW for BC production [55,56]. Based on similar literature reports, BC production is species and strain dependent. Evidently, the results of the current work confirmed the ability of *A. xylinum* to use CW hydrolysate under three different fermentation schemes. Hydrolysates derived from 11.25 (Hydrolysate A) and 7.5 U/mL (Hydrolysate B) crude β-galactosidase, respectively, were used to evaluate BC production, and the results are presented in Table 1. Different nitrogen concentrations were used, based on previous observations where elevated levels of nitrogen content induced cell proliferation at the expense of BC production [32]. As it can be seen in Figure 5a, the consumption of 13.91 g/L of glucose and 224.79 mg/L of FAN resulted in the production of 7.05 g/L BC (Hydrolysate A). Hydrolysate B followed a similar trend with respect to glucose consumption rate. The consumption of 13.41 g/L of glucose and 132.64 mg/L FAN, resulted in 5.78 g/L of BC production (Figure 5b). However, it is worth noting that in both experiments a considerable amount of sugars remained unfermented by *A. xylinum*. Therefore, a third treatment was deployed using diluted hydrolysate (hydrolysate C) (1:1 CW:H_2O) in order to evaluate the BC production yield on approximately 25 g/L total sugar content and 260 mg/L FAN concentration (Figure 5c). In fact, in these experimental conditions, almost complete glucose consumption was attained along with the consumption of 114.94 mg/L FAN, achieving a final BC concentration of 5.59 g/L.



(**a**)



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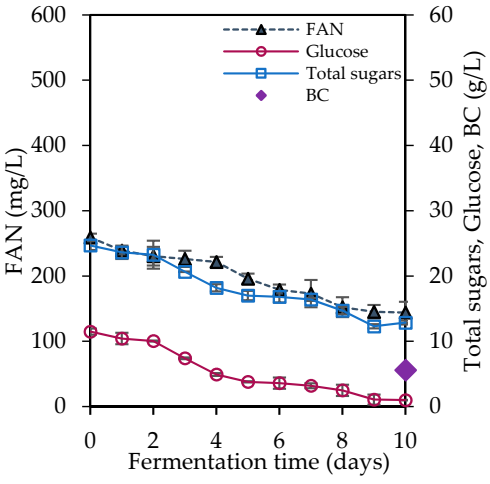

(**c**)

Figure 5. Bacterial cellulose (BC) production and kinetics of sugars and free amino nitrogen (FAN) consumption, using different cheese whey (CW) hydrolysates. (**a**) Hydrolysate A; (**b**) Hydrolysate B; (**c**) Hydrolysate C.

Table 1. Experimental schemes of cheese whey and cheese whey hydrolysates fermentation by *A. xylinum*.

Fermentation Media	Initial Total Sugars (g/L)	Initial Glucose (g/L)	Residual Glucose (g/L)	Initial FAN (mg/L)	FAN Consumption (mg/L)	BC Production * (g/L)	BC Productivity (g/L/d)
Hydrolysate A	45.04 ± 2.60	23.31 ± 0.77	9.40 ± 0.24	520.05 ± 0.34	224.79 ± 8.39	7.05 ± 0.14 ^{A}	0.71
Hydrolysate B	45.80 ± 0.77	22.35 ± 0.46	8.93 ± 0.19	331.36 ± 12.96	132.64 ± 5.21	5.78 ± 0.35 ^{A,B}	0.58
Hydrolysate C	24.68 ± 0.69	11.48 ± 0.48	1.00 + 0.08	259.25 ± 5.70	114.94 ± 4.73	5.59 ± 0.22 ^{B}	0.56
Cheese whey A	50.00 ± 1.22	2.51 ± 0.23	-	56.00 ± 3.35	19.49 ± 2.78	0.58 ± 0.01 ^{a}	0.06
Cheese whey B	24.45 ± 1.03	1.28 ± 0.10	-	22.98 ± 5.70	22.98 ± 0.00	0.71 ± 0.05 ^{a,b}	0.07
Cheese whey C	24.29 ± 1.18	1.39 ± 0.14	-	250.00 ± 10.06	81.33 ± 4.65	1.07 ± 0.09 ^{b}	0.11

* Different letters (A, B, a, b) within each group (hydrolysates and cheese whey) indicate significant differences ($p < 0.05$). FAN: free amino nitrogen; BC: bacterial cellulose.

The above results are in accordance with other studies describing the utilization of several monosaccharides and disaccharides as carbon sources to generate BC by various *Acetobacter* spp. strains. Semjonovs et al. [55] reported a high BC yield with CW hydrolysate (20 g/L reducing sugars) as the sole carbon source using the strain *Komagataeibacter rhaeticus* P 1463. Additionally, Salari et al. [57] recently referred to a BC production of 3.5 g/L within 14 days of fermentation by *Gluconacetobacter xylinum* PTCC 1734 in static cultures, using an equimolar glucose/galactose mixture from hydrolyzed CW. In all the conducted experiments, a considerable increase in BC production was observed when compared with the results obtained by media with lower amount of FAN concentration. On the other hand, BC production levels by unhydrolyzed whey were quite close to those previously reported [58]. More specifically, as it is presented in Table 1, *A. xylinum* consumed 19.49 mg/L of FAN, producing 0.58 g/L of BC in unhydrolyzed CW (cheese whey A) (Table 1). Likewise, significant differences ($p < 0.05$) on BC production were observed, when different fermentation media were applied, whereas significantly higher concentrations were produced using all types of CW hydrolysates, compared to sole CW (Table 1).

Recently, Kumar et al. [59] demonstrated the production of 1.4 g/L of BC under static culture conditions in whey medium by *Acetobacter pasteurianus*. The formation of BC in these cases is mostly due to the presence of several other compounds such as the residual carbon present in the initial inocula. In addition to this, higher BC production was observed using diluted CW, which is consistent which similar studies [60]. In specific, *A. xylinum* produced 0.71 g/L and 1.07 g/L BC, when diluted CW (cheese whey B) and diluted CW supplemented with yeast medium (cheese whey C) were, respectively, applied (Table 1). In general, lactose as a sole carbon source is reported as a weak substrate for BC production leading to 0.04–0.07 g/L [39,61], while BC production by unhydrolyzed CW is recorded slightly higher ranging from 0.15 to 0.78 g/L [58,60]. Our results (using unhydrolyzed CW) are in agreement with those previously reported, whereas BC production was significant higher using CW hydrolysates. Overall, in this study, high production of BC was achieved using CW hydrolysates compared even to BC production using conventional synthetic HS medium. These findings are exceptionally promising pointing out potential for a cost-effective bioprocess.

3.4. Technological Consideration of the Study

The principal target of this study was the development of a holistic exploitation approach for cheese whey, that will engage with sustainability and generate value-added products via the aligned food waste reduction and by-product streams treatment, as cornerstones of the circular economy concept. Likewise, an efficient fungal-based, two-stage bioprocess was employed to produce a nutrient rich feedstock for subsequent upstream bacterial bioconversions. Cost effective production of crude enzymes, without further purification steps was undertaken using food industry by-products, specifically cheese whey. The significant hydrolytic activity of this novel biocatalyst was demonstrated, leading to the formulation of a suitable feedstock for bacterial cellulose (BC) production. The results of our study confer an insight for the fermentative production of BC using whey

lactose hydrolysates, which effectively sustained the nutrient requirements of *A. xylinum*, displaying high production yields. Evidently, enhanced feasibility could be established through the development of suitable bioprocesses to mediate BC production costs via the replacement of conventional fermentation media. The consolidated bioprocess presented hereof is currently further extended within the concept of holistic refining of cheese whey streams (lactose and protein). In particular, in our forthcoming research, novel probiotic starter cultures will be developed, and BC generated in this study will be implemented as a carrier for lactic acid bacteria starter to be reintroduced into dairy products, thereby closing the loop. Ultimately, the combined proposed approach conforms to the pillars of circular bioeconomy, encompassing environmentally benign processes, zero waste generation in parallel with novel food product development and potential health benefits.

4. Conclusions

The results of the present study indicate the successful development of a novel cheese whey valorization approach within the concept of circular bioeconomy. More specifically, a two-stage operation was established to generate crude enzymatic consortia via fungal solid state fermentations with *A. awamori*. Fermentation conditions were optimized and a novel biocatalyst was effectively secreted, and subsequently implemented to hydrolyze whey lactose formulating a nutrient substrate for fermentative bioconversions. BC production was conceptualized as a transitional compound for subsequent functional food formulations, along with the protein fraction to complement sustainability and circularity of the process.

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