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Biotransformation of food waste to starter culture biomass: An investigation of antibiotic resistance-free lactic acid bacteria from dairy and household food waste

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Abstract
The food wastage due to household and industrial processes is estimated to be hundreds of tons representing environmental and economic issues. Lactic acid bacteria (LAB) led biorefinery processes of industrial interest are considered sustainable valorization techniques for nutrient-rich food organic waste. However, this food waste can be a major dissemination channel for transmitting antibiotic resistance (ABR) from non-pathogenic strains to the consumer. Considering the importance of non-antibiotic resistance (ABR) LAB as microbial starters, this study focused on isolation and characterization of non-ABR LAB from the household and dairy industrial food waste and assess its suitability for biomass production of the bacteria. Among 75 total LAB isolates, around 60% from hostel food, 33% from household waste and 30% from dairy waste were identified as non-ABR LAB against 7 antibiotics examined. Interestingly, ABR incidence of dairy waste is much higher than that of household waste. Most LAB strains were resistant to Tetracycline and Neomycin and showed a wide range of Vancomycin resistance, indicating serious threats to the food waste like environment. Likewise, the susceptibility of LAB to β-lactam group antibiotics was more than 10% across the food waste samples. This study emphasizes the antibiotic resistance-free lactic acid bacteria from dairy and household food waste biotransformation to starting culture biomass.

Keywords: waste utilization, ABR, non ABR strains, fed batch fermentation, LAB

1. Introduction
Any part of food that is excluded, regardless of its potential concentration of nutrients with a high value, is characterized as food waste (Hartikainen *et al.*, 2018) [13]. In recent decades, the food waste due to processing and distribution steps has been raised and globally, an estimated one-third of the food intended for human consumption is lost every year. It is indicated by Food and Agricultural Organization (FAO) that 33% of food delivered for human utilization is lost along with the food inventory network internationally and further expected to expand over the next 25 years in Asian countries. Between 2005 and 2025, the yearly volume of urban food waste in Asian countries might climb up to 416 million tons. (Uçkun Kiran *et al.*, 2014) [28]. Through microbial interventions, sustainable food waste management technologies are essential to mitigate the negative environmental impact of household food waste. Likewise, dairy waste disposal, including the dairy processing by-products rich in organic compounds, represents a major threat to the environment. Therefore, the interest in finding alternative green valorization techniques for effective household and dairy waste biorefinery increases according to sustainable development goals 12.3 (FAO).
Considering the rising food waste issues, the modern challenge lies in the efficient management of minimizing the food waste and solutions to utilize the food waste rich in high-value components according to a circular economy strategy. The major components of food waste include starchy polymers, lignin, proteins, lipids, organic acids, and other inorganic constituents. Total sugar and protein contents in food waste are in the range of 35.5–69% and 3.9–21.9%, respectively (Li & Yang, 2016) [17]. Kitchen waste is a carbohydrate - rich organic matter with high moisture that can easily biodegradable (Dodamani & Ravi, 2015) [11]. The breakdown of glycoside linkages during carbohydrate hydrolysis in food waste may result in polysaccharides as oligosaccharides and monosaccharides, which are more fermentable. Meanwhile, dairy waste has a considerable range of lactose, protein, and fat in the range of

250 to 930 ppm, 210 to 560 ppm and 35 to 500 ppm, respectively (Belletti *et al.*, 2009) [5]. As a result, household waste and dairy waste are excellent nutritional sources to use as a substrate. However, the challenge is optimizing the carbon to nitrogen ratio because its metabolism is tightly linked to the cultivation of bacteria in a fermentation medium (Śliżewska & Chlebicz-Wójcik, 2020) [27].

Lactobacilli are an essential component of the intestinal microbiota of mammals. They have been used safely as food and feed supplements for a long time. (Ammor *et al.*, 2007) [3]. Lactic acid bacteria (LAB) are gram-negative, aero tolerant, fermentative, and GRAS status bacteria widespread in natural food such as vegetables, fruits and dairy and used as a starter culture in a variety of fermented foods. Though the United States- Food and Drug administration has awarded "Generally Recognized as Safe (GRAS status)" to LAB, it is essential to fulfil the criteria of absence of antibiotic resistance (ABR) genes against a wide class of antibiotics of clinical importance. However, in the last decade, in the light of recent scientific interventions, increasing concerns raised issues about the safe use of LAB due to the possible role of antibiotic resistance (ABR) gene reservoir. The occurrence of transposons, horizontal gene transfer and conjugated plasmids like phenomena in LAB is a raising concern pertaining to the probiotic applications of the bacteria. Though the clinical (Clementi & Aquilanti, 2011) [7]. and environmental microbes (Vignesh *et al.*, 2012, 2016) [31, 30] are investigated more for the presence of ABR, recent studies focus on food-associated microbes and their potential risk to human health. Meanwhile, aerobic fermentation is a viable technique in the food and dairy industries, where most of the energy and organic compounds are utilized by microbial culture for their growth (McLAUGHLIN, 1946) [22]. The utilization of renewable biomass, especially food wastes can help to improve bio remedial measures to serious environmental threats and resource wastage (Liu *et al.*, 2016) [20]. In particular, the fed-batch fermentation is beneficial to enhance biomass cultivation of bacteria and prevent bioconversion inhibition due to a high initial substrate concentration (Hewitt & Nienow, 2007) [14].

In this work, aiming at valorizing the household waste and dairy waste, non-ABR *Lactobacillus* sp. colonizing the food waste was isolated. The food waste was further optimized as a growth medium for mass cultivation of those non-ABR LAB.

2. Material and Methods

2.1 Sample collection and materials

The food sample (curd, curd rice, and boiled rice) and the household waste (chicken, fish, chapati, dal, and fermented rice products such as idli, dosa) were collected from a house, and the dairy waste (effluent waste) was collected from the dairy industry in and around the Thanjavur district, Tamil Nadu, India. The food sample was collected, blended, and directly stored, whereas food waste at selected locations was allowed to be in the waste collection bins for 24 hours and then collected. The solid and liquid waste were collected in sterile containers and immediately stored in the icebox, carried to the laboratory, and stored in a refrigerator prior to 4 °C until further analysis. The chemicals, reagents, and microbial media used in this study were hi-grade purchased from Himedia Pvt. Ltd, Mumbai, India. Sterile ultrapure Milli Q water was used in the entire study.

2.2 Isolation and identification of LAB

About 1 g of the sample was suspended in 100 ml of sterile saline solution and mixed through a rotary shaker for 30 min. Then, the serially diluted sample was inoculated into De Man, Rogosa Sharpe (MRS) agar (Merck, Darmstadt, Germany) agar (McLAUGHLIN, 1946) [22]. The plates were incubated at 37±2 °C for 24 hrs. After plating, the milky white, translucent white, yellow, pale-yellow colonies were randomly picked at higher dilution, purified by successive streaking, and transferred to MRS broth. The bacteria isolated from the selected media were referred to as like organisms (LO) of the lactic acid bacteria group (Vignesh *et al.*, 2015) [29] and were further confirmed by biochemical characterization assays. The bacterial isolates were stored at 4 ºC on respective media for short-term maintenance and 40% glycerol at -80 ºC for long-term storage.

2.3. Identification of antibiotic-resistant LAB

The antibiotic resistance of the isolated LAB strains was performed through Kirby-Bauer disk diffusion method in accordance with CLSI document M2-A9 suggestions. Norfloxacin (10 mcg), Chloramphenicol (30 mcg), Penicillin G (10 mcg), Erythromycin (15 mcg), Tetracycline (30 mcg), Neomycin (10 mcg), Vancomycin (30 mcg) were the antibiotics from the classes Quinolone, Phenicol, ß-lactam, Macrolide, Tetracycline, Aminoglycoside, Glycopeptide respectively were utilized for this study.

Table 1: Antibiotics, their classes and dosage

Antibiotic	Class	Dosage (µg)
Norfloxacin	Quinolone	10
Chloramphenicol	Phenicol	30
Penicillin G	ß-lactam	10
Erythromycin	Macrolide	15
Tetracycline	Tetracycline	30
Neomycin	Aminoglycoside	10
Vancomycin	Glycopeptide	30

After strains were activated on MRS agar, they were cultivated in Mueller Hinton Agar and then with the antibiotic disc by rotating the plate for every 60° to achieve even bacterial growth using a sterile cotton swab. After incubation (37 ºC, 24h), the antibiotic resistance of the LAB strain was deciphered based on the proposals of the National Committee for Clinical Research facility Standards for antimicrobial vulnerability tests (NCCLS, 1998) by measuring the inhibition zone diameter around the discs (Vignesh *et al.*, 2016) [30].

2.4. Preparation and optimization of food waste as a fermentation medium

To utilize the food waste (household and dairy waste in this study) to produce culture biomass, the food waste medium was optimized for LAB growth. The LAB with no ABR (VPL) was selected for cultivating in a food waste medium. The food waste was pulverised separately, blended into a thick paste, centrifuged at 9000 rpm for 10 min, and stored at 4 °C. Moisture content in the sample was also determined (Vera Zambrano *et al.*, 2019). The anthrone technique was used to determine the amount of carbohydrates (Loewus, 1952). Protein and reducing sugar content were determined using Kjeldahl and Nelson-Somogyi techniques following AOAC methods. The biomass was determined by dry weight measurement (Baur & Ensminger, 1977) [4].

To obtain the desired C/N molar ratio comparable to the culture medium (E. g. MRS Agar), the food waste medium was optimized through varying substrate concentrations. The samples were blended and centrifuged at 8000 RPM for 15 minutes to get a liquid food waste medium. Five different compositions were screened to identify a suitable one for further experiments. All media compositions were consisting of pH 6.5 ± 0.5 before sterilization at 121 ºC for 15 min. VPL was cultured in an optimized medium at 37 degrees Celsius for 48 hours and the OD values were noted to observe the microbial growth after 24 and 48 hours consecutively.

2.5. Fed batch fermentation for biomass production of VPL strain

The biomass cultivation of non-ABR LAB using food waste medium in a fed batch fermentation process was carried out in a view to their industrial application. The fed batch fermentation was carried out in 500 ml Erlenmeyer flask and the initial working volume of 250 ml and pH and temperature of 6.5 ± 0.5 and 35 ± 0.5 degrees Celsius. The substrates used for batch fermentation were household waste, dairy waste and combination of household and dairy waste. The mixture was prepared by adding 40g waste in 200 ml of distilled water then centrifuged at 8000 RPM for 15 minutes. The supernatant was collected and stored in sterile containers. Total 100ml volume was made as a substrate by addition of 75 ml of waste sample and 25 ml of distilled water for household and dairy waste sample. Also, the combination of substrate included 37.5 ml of household waste, 37.5 ml of dairy waste and 25 ml of distilled water. The prepared substates were autoclaved and cooled. 1 ml of microbial strain was added to the substrates by maintaining aseptic conditions. 3 ml of feed was added to the substrate after every 6 hours until 96 h. The feed comprises of the basic substrate with addition of soya protein (18 ml substrate + 0.1 g soya protein). UV spectrophotometric readings were taken after every 6 hours until 96 h. In addition to that, sample collected from 24th hour was plated on nutrient agar medium to ensure the growth rate of microbial culture.

2.6. Data analysis

All the experiments were conducted in triplicate and the data were represented as mean ± standard deviations. One way Analysis of variances (ANOVA) was performed in SPSS version 25 for outcomes obtained from fed batch fermentation experiments.

3. Results and Discussion

3.1. Isolation of LAB and identification of ABR incidences in food waste origin

The hostel food, household waste and dairy waste were perceived in this study as low-cost source for cultivation of LAB to effectively valorize the food waste as well as for commercial cultivation of starter culture suitable for food industries. Although the LAB is considered "generally recognized as safe" (GRAS), the ability of the bacteria to exchange genes with the colonized environment for their survival give rise to threats and reduces the efficiency of the antibiotic therapies (Klose *et al.*, 2014) [16]. Hence, the selection of potential probiotic strain lacking ABR genes is considered primary for commercial applications. Based on the food waste fermentation results obtained using LAB isolated from the hostel and dairy sources, the promising potential of the single or mixed food waste medium valorization. Around 75 LAB were isolated from hostel food (25), household waste (25) and dairy waste (25) samples. Colonies were isolated on the basis of morphology on the MRS agar, gram staining as well as physicochemical tests such as catalyst test, oxidase test.

The prevalence of agricultural antibiotics in daily food products leading to the selection of antibiotic resistant bacteria. Further, the ABR study is a critical criterion for starter culture selection as natural antagonists of potentially dangerous bacteria (Georgieva *et al.*, 2015) [12]. Data of ABR patterns tested against seven different antibiotics is provided in Table 2. More than 15 mm diameter was observed in 18 of the strains isolated from hostel food, whereas 10 and 9 strains were observed in household waste and dairy waste respectively. Non-ABR, or beneficial cultures, were defined as strains with an inhibitory zone greater than 15 mm. (Hi-media zone size interpretative chart). In food sample, around 60% were non-ABR, whereas in the household waste it was around 33% and in dairy waste around 30%. The agri-food sludge acts as ABR reservoir, due to the cross contamination of antibiotic polluted manure and soil (Lipsitch *et al.*, 2002) [19]. Likewise, the LAB of food waste showed ABR to 66% in case of household waste and 70% in case of the dairy waste. The LAB of dairy products acquires ABR genes from the raw milk through plasmids, transposons and horizontal gene transfer like phenomenon (Mathur & Singh, 2005) [21].

This study explored the ABR of LAB isolated out of this study to 7 classes of antibiotics and the isolates of hostel food showed susceptibility to β-lactam (30%), Macrolide (13.3%), and Phenicol (10%) remarkably. Similarly, the susceptibility percentage of LAB from household waste was higher against vancomycin (13.3%) followed by Macrolide (6.6%). (Sen *et al.*, 2016) [26] reported that the waste from fermented food that are not thermally treated before are in general play a key role as a vehicle for ABR bacteria since they are in connection with animal or human microflora and fecal contaminants. All isolates of food waste samples have Neomycin resistance, whereas most of the isolates are susceptible to Penicillin and Erythromycin in this study. Natural resistance of LAB to Aminoglycoside antibiotic groups were documented by many authors (Davies *et al.*, 1971) [9] (Rojo-Bezares *et al.*, 2006) [25]. Another interesting finding of this study is vancomycin resistance (more than 80% of the LAB isolates are resistant to this glycopeptide antibiotic, which should be investigated for transmission to pathogenic bacteria. (Ammor *et al.*, 2007) [3] have cited the vancomycin resistance of LAB family such as *Leuconostoc*, *Lactobacilli* and *Pediococci* which becomes a property to differentiate gram-positive bacteria. The very high frequency of mutations in lactobacilli might be one fact behind the growing antibiotic resistance among the LAB organisms (Mathur & Singh, 2005) [21]. In another study experimented the ABR of LAB from cheese sample, none of the strain were completely susceptible to the many of the antibiotics tested which indicates a multiple resistance due to selective pressures (Herreros *et al.*, 2005).

Table 2: Antibiotic Resistance and susceptibility in selected hostel food, household waste and dairy waste

Antibiotics	Classes	Hostel food n= 25		Household waste n= 25		Dairy waste n= 25	
		N	%	N	%	N	%
Norfloxacin (10mcg)	Quinolone	0	0	1	3.3	2	6.6
Chloramphenicol (30mcg)	Phenicol	3	10	0	0	2	6.6
Penicillin G (10mcg)	β-lactam	9	30	3	10	2	6.6
Erythromycin (15mcg)	Macrolide	4	13.3	2	6.6	3	10
Tetracycline (30mcg)	Tetracycline	2	6.6	0	0	0	0
Neomycin (10mcg)	Aminoglycoside	0	0	0	0	0	0
Vancomycin (30 mcg)	Glycopeptide	0	0	4	13.3	0	0
Total susceptible strains		18	60	10	33.3	9	29.8
Total resistant strains		7	40	15	66.7	16	70.2

3.2. Optimization of food waste medium for enhanced bacteria biomass production

In view of effective food waste valorisation, the selected food waste samples for LAB isolation were optimized to increase the yield of bacterial biomass. Extrinsic (Temperature) and intrinsic factors (pH and nutrient medium) affecting the bacterial growth in food waste medium were optimized with preliminary trials. The food waste chemical composition was analysed before fermentation reaction, in order to access the nutritional composition and losses during autoclaving the sample by Maillard reactions like processes. The household waste had the following chemical composition: 3.08 (d.b) mg/g reducing sugar, 0.35 mg/g (d.b) protein with an initial pH of 4.38, whereas, the composition of dairy waste is 0.07 (w.b) mg/ml of reducing sugar and 0.007 (w.b) mg/ml of protein with pH value of 5.56. Carbohydrates in food waste are abundant in amount (Abedi & Hashemi, 2020) [2] in comparison to nitrogen content. In the conducted study, importantly, carbon as to nitrogen ratio present in raw substrates was found 1:2. Besides, considering the importance of nitrogen for microbial protein synthesis, the carbon/ nitrogen ratio was tuned according to the original composition of carbohydrates and proteins in addition to the pH.

Aiming at evaluating the suitability of the food waste medium for LAB cultivation, the carbon/ nitrogen ratio, was tuned based on the original composition of carbohydrates and proteins to maximize the yield of LAB biomass. The household waste was finely grinded and dairy waste was thoroughly homogenized before added to sterile distilled water in the percentage ranging from 20 to 100%. The following parameter were altered: amount of food waste substrate and concentration of supplement added. Selected non-ABR LAB strain was inoculated in prepared substrates. The cell density of the selected LAB species was measured after incubation of all the formulated food waste medium compared to the nutrient media commonly used to propagate bacteria. UV spectrophotometric reading at 630 nm after each 6 hours was noted on 24h and 48h. In addition to that, plating is also done after fermentation to ensure the growth rate of inoculated LAB strain.

The bacterial biomass difference observed on 24 h and 48 h hour differ significantly, and the growth was substantial at 48 h. After 24 h of incubation at 37 ºC, the bacterial biomass was observed with different combinations of food waste medium using OD 630 nm value. Overall, the LAB density was higher in 1:1 substrate concentration (50% food waste medium), compared to other food waste medium compositions (Fig. 1). Previous literatures also suggested the use of mixed waste biomass in equal concentrations to setup food waste refinery plans to obtain maximum value-added components that works in any environmental conditions (Lin *et al.*, 2013; Nizami *et al.*, 2017) [18, 23].

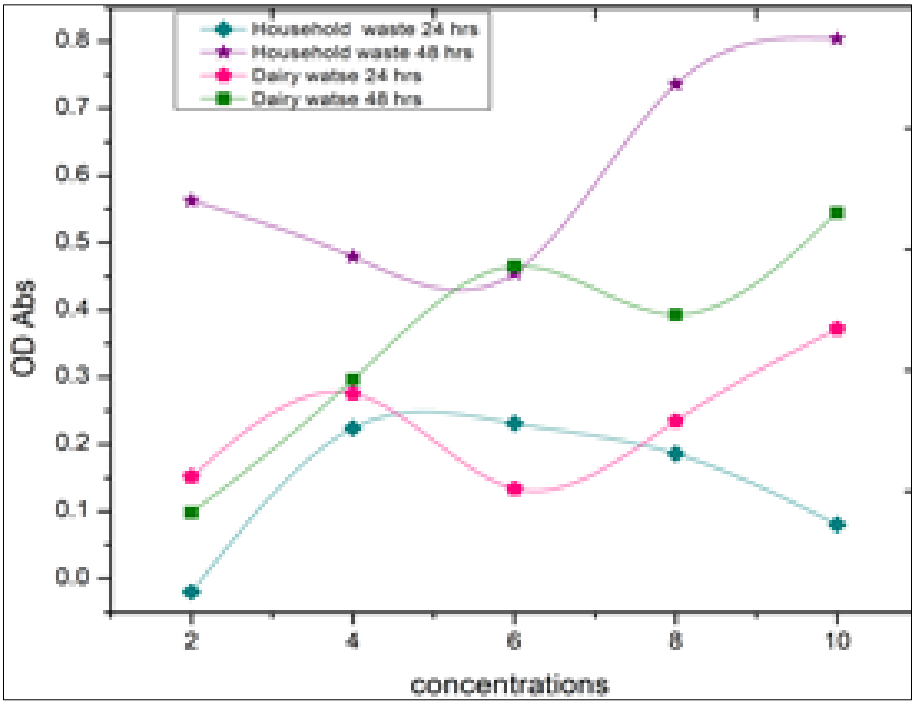

Fig 1: Optimization of food waste substrate concentrations for maximum biomass production

Notwithstanding, media obtained with food waste sample containing 1:1 proportion of carbon to nitrogen ratio was selected as fermentation substrate in terms of final LAB biomass cell density. The combination of substrate and proportion is secured based on the previous optimization study.

3.3. Fed batch fermentation for cultivation of starter culture biomass

The fed batch fermentation has been recommended for industrial process to eliminate the inhibition of substrates and to maximize the culture density. In this study, about 3 ml of formulated food waste (1:1) solution containing individual substrates as well as combined substrate were added to extend the exponential growth phase of bacteria at high rate and in turn to maximize the cell density. To maximize the microbial growth, all the fermentation medium was supplemented with 0.1g of soya protein every 6 hours once to boost the nutrient content of the substrate. The biomass production was monitored for 96 h after 12 h of incubation period. The biomass production reached a maximum OD value of 1.59 in household waste, 1.84 in dairy waste and 2.119 in combined waste in comparison to the standard nutrient broth medium (Fig. 2). We found, biomass production in combined household and dairy waste grew the most, followed by dairy waste, and least in the household waste. The fed-batch fermentation have shown that the LAB biomass production is promising, as cited in the previous research works in agri-food waste substrate for fermentation by LAB to obtain high biomass yield (Hewitt & Nienow, 2007) [14].

In fact, the high LAB biomass out of this study supports the fact that no specific pre-treatment of food waste is required before fermentation except the routine griding and homogenization. Moreover, the biomass yield in mixed food waste was higher than the single substrate, confirming the non-ABR LAB strain out of the food waste medium can be utilized and grow on different carbon sources.

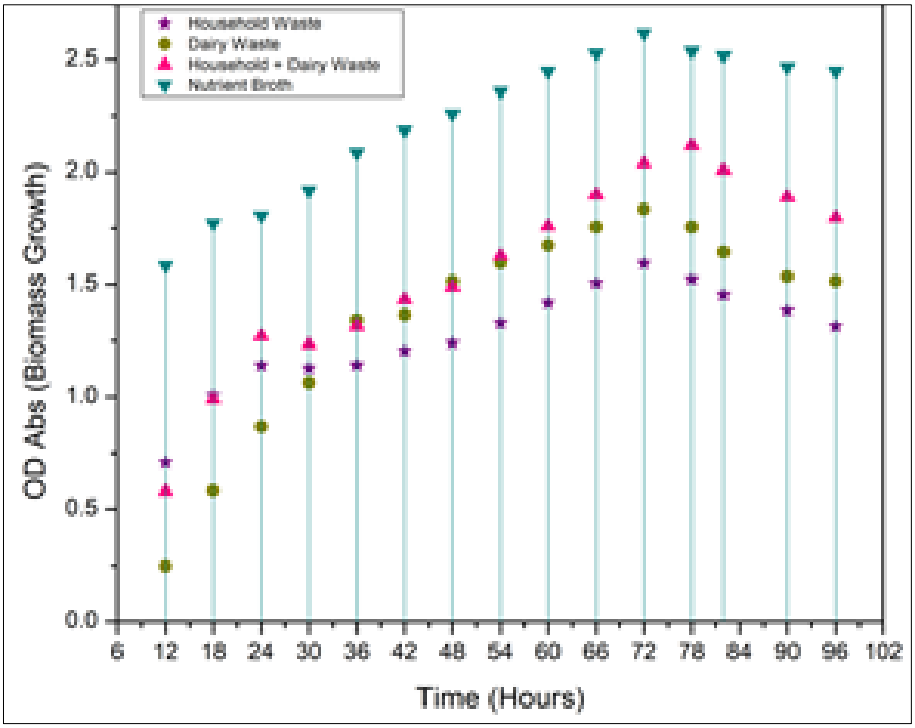

Fig 2: Comparison of mass production with nutrient broth

In a study conducted by (Costa *et al.*, 2020) [8] lactic acid production from Agri-Food Residues the *L. casei* was shown to exhibit higher growth in mixed biomass than the single waste biomass emphasizing the importance of multiple carbon source for high yield of starter culture.

The nutrient broth medium was used as a reference substrate for testing the efficacy of food waste fermentation through the isolated LAB culture. From the results of this study, it emerged that household waste and dairy waste when combined gave remarkable difference in the bacterial biomass production and also comparable to a standard nutrient medium.

3.4. Trends in the concentration of nutrients after food waste fermentation

Overall, the LAB biomass yield obtained in the fed-batch fermenter from different food waste, is high and good as reported by (Dedenaro *et al.*, 2016; Kaur *et al.*, 2019; Wang *et al.*, 2017) [10, 15, 32] proving the possibility to valorize the food waste and to convert that to high value biomass at the lowest cost of the substrate. Based on the proximate analysis results of fermented food waste medium, the carbohydrate, protein, and fat were: 53.35, 5.54 and 5.06% respectively in household waste, 49.69, 10.61 and 10.10% respectively in dairy waste and 52.59, 11.80 and 6.74% respectively in combined waste.

The purpose of adding soy protein was to verify the effect of increasing the nitrogen source on the biomass production. Based on the results obtained, we concluded that the enhancing the nitrogen content of the food waste have a positive impact on bacterial biomass yield and the mixed waste can boost the nutrient balance for microbial growth.

Many authors emphasized the role of nitrogen in a fermentation medium (Abbasiliasi *et al.*, 2017; Bouguettoucha *et al.*, 2011) [1, 6]. Since the food waste is a complex nutrient medium which are generally rich in minerals, vitamins and simple sugars, addition of an extra

nitrogen pushes the biomass production from the basal medium and makes ways for the valorization of heterogenous food waste medium.

Though both the batch and fed batch fermentation are reported to increase the biomass yield, fed-batch fermentation is the effective system that guarantee high culture density (Othman *et al.*, 2017) [24]. Meanwhile, in this study, after 48 h of intermittent addition of nitrogen source (soy protein) the nutrients tend to deplete after 72 h of fermentation once the nutrients start depleting out of the food waste composition. Hence, it can be elucidated that knowledge-based strategies are required to make use of a fermentation medium, particularly if the substrate is from multiple sources.

4. Conclusion

The study results that non-LAB based biomass yield utilizing food waste, and the optimized food waste medium represents a realistic option for reuse and valorisation of household food waste and dairy effluents. Meanwhile, it cannot be ignored that the presence of ABR in almost of the LAB isolates in a food waste medium fuel the debate about their utility in food processing industries and the possibilities of its transmission to other clinical strains through food chain These results expand our knowledge on the possible role of food waste-based LAB as a source for disseminating ABR through the food environment.

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