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Research Article

Oleaginous yeast isolated from dairy foods as a high lipid and protein supplement for animal feed

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Abstract

Microbial cell factories based nutraceuticals provide an excellent source of high nutritive value feed at very low cost for dairy and small animal stocks. Two yeast isolates, *Candida zeylanoides* CHV and *Candida metasilopsis* IY, from dairy products were studied for their efficiency in the generation of biomass and cytoplasmic lipid accumulation. The growth rate and lipid production from our two oleaginous yeasts, along with the reference strain *B. adeninivorans* MTCC 2516 was observed in whey-based media formulation. The highest lipid content of 77.92±7.79% of dry cell mass was harvested from *B. adeninivorans* MTCC 2516 followed by CHV (76.17±8.43%) and IY (75.79±3.06%) isolates in early stationary phase (ES). The relative conversion of whey into % lipid, carbon and protein content were observed. The most efficient conversion of whey media in value added components was done by yoghurt isolate *Candida metasilopsis* IY that exhibited highest relative 54% lipid content followed by 41% carbon content as well as lowest 1% ash content of its dry cell mass in late stationary phase (LS). However, the relative highest protein content was recorded in *Candida zeylanoides* CHV at ES (35%) and LS (31%). Hence, the efficient conversion of whey-based feedstock into food grade oleaginous yeasts in our study provides a more acceptable option in human nutrition, animal feed and aqua feed.

Keywords: Animal feed supplement, Oleaginous yeasts, Single cell oil, Single cell protein, Whey bioprocessing

Highlights

- Two lipid accumulating yeast strains were isolated – *Candida zeylanoides* CHV and *Candida metasilopsis* IY from cheese and yoghurt.
- The whey media formulation used in the study furnished better growth of oleaginous yeasts and offers a cost-effective alternative to commercial media for biomass production.
- The most efficient conversion of whey media in value-added components was done by yoghurt isolate *Candida metasilopsis* IY, which exhibited the highest relative 54% lipid content.
- The two yeast isolates are safe to use as single cell oil and single cell protein in animal feed supplementation.

INTRODUCTION

Oleaginous microorganisms, sometimes called single cell oils (SCOs), are being widely used for the industrial production of microbial lipids. All microorganisms are composed of lipids usually comprising around 6 to 8% (w/w) of their dry cell weight. Microorganisms producing more than 20% (w/w) of their dry cell weight as lipids are termed oleaginous (Abeln and Chuck, 2021). Because of their lower environmental footprints in terms of land, water, and carbon emissions, lower labour requirements, increased resilience to climate change, shorter production cycles, flexibility, and adaptable processes, oleaginous microorganisms are a desirable alternative to traditional lipid sources like plants, fish, and animals.

Microbial culture conditions significantly influence lipid content and biomass production, alongside the selection of appropriate oleaginous yeast strains (Jones *et al*., 2019). Under nutrient-deficient conditions, these yeasts have shown a remarkable ability to accumulate lipids, often exceeding 70% of their biomass. This capacity underscores their potential in lipid production for various applications, including biofuel and biotechnology industries. Understanding and optimizing culture parameters such as carbon-to-nitrogen ratio, pH, temperature, and agitation are essential for maximizing lipid yield while balancing biomass production, offering promising avenues for sustainable lipid production (Taskin *et al*., 2015).

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Cheese/paneer whey is one of the most readily available culinary waste in the Indian dairy industries. Approximately 197.44 million tonnes of whey are produced worldwide from all cow milk cheeses, making whey a significant by-product of the cheese business (Shelke *et al*., 2022). About 10 L of cheese/paneer whey are produced for every kg of cheese/paneer production. Lactose (45-50 g/L), proteins (6-8 g/L), lipids (4-5 g/L), and minerals primarily like calcium, potassium, and phosphorus make up the majority of the contents of cheese/paneer whey.

In this context, the microorganisms for conversion of these organic wastes into value added products like single cell protein or single cell oil offer a sustainable nutritional source for various applications. Using oleaginous yeasts to value food waste presents a viable way to apply biotechnological processes in a circular economy competition. The present study explored oleaginous yeast isolated from dairy products for microbial biomass, lipid, and protein conversion from waste liquid whey.

MATERIALS AND METHODS

Yeast strains: One reference strain, *Blastobotrys (Arxula) adeninivorans* MTCC 2516, was obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh. The yeast isolates were collected from fermented and non-fermented dairy products by plating on Potato dextrose agar (PDA) containing chloramphenicol (0.05 g/L). Isolated yeast colonies from PDA-chloramphenicol agar along with reference strain *Blastobotrys adeninivorans* were screened for the production of cytoplasmic lipid granules using the Sudan black B staining (30% w/v in 70% ethanol) using the protocol given by Thakur *et al*. (1988). Two yeast isolates from cheese and yoghurt samples, showing the presence of cytoplasmic lipid granules, were identified as *Candida zeylanoides* CHV and *Candida metasilopsis* IY, respectively, using MALDI-TOF MS (Bruker MALDI Biotyper) at Microbial Type Culture Collection (MTCC), Chandigarh.

Gelatinase test: The safety aspect of two strains, namely CHV and IY, along with the standard reference strain *B. adeninivorans* MTCC 2516, was assessed by evaluating gelatinase activity and haemolytic activity by streaking the pure culture on gelatin agar and blood agar as per the method described by Victor *et al*. (2011). The gelatinase test was performed on nutrient gelatin agar (nutrient agar containing 3% gelatin). Two microliter of an active log phase culture was spot-inoculated on nutrient gelatin agar plate. The plate was incubated aerobically for 48 h at 25°C, after which it was flooded with saturated ammonium sulfate solution.

Haemolytic activity test: Haemolytic activity was assessed on a blood agar plate. Active log phase cultures were streaked on blood agar and incubated aerobically for 48 h at 25°C. After incubation, plates were observed for the presence of a clear or greenish zone around the yeast culture growth.

Standardization of whey-based media for yeast cultures: The cheese whey was deproteinized and supplemented with various salts (KH_2PO_4 30 g/L; NaH_2PO_4, 3 g/L; $MgSO_4$, 3 g/L; $CuSO_4 \cdot 5H_2O$, 0.006 g/L; $MnSO_4 \cdot 5H_2O$, 0.004 g/L; $ZnSO_4 \cdot 7H_2O$, 0.002 g/L) as described by Taskin *et al*. (2015) and, Vyas and Chhabra (2019). Finally, stabilizer Carboxy Methyl Cellulose (CMC) 0.05% (w/v) was added to the whey media formulation and autoclaved for 15 minutes at 121°C.

Growth of isolated yeasts in whey media: The growth of yeast in whey media and standard YEPD media was compared using growth curve analysis of cultures in both media. The yeast cultures were inoculated in 100 mL whey media and YEPD media and incubated at 25°C temperature up to 88 h. The cell number in the medium during every 4-hour interval was determined by aseptically taking out 1 mL of the sample and plating the appropriate dilution on a PDA-chloramphenicol media plate.

Lipid production: The selected yeasts were taken for the analysis of lipid production using whey based media during early and late stationary phase of the growth. The method proposed by Bligh and Dyer (1959) was followed for gravimetric assessment of lipid production. Briefly, 10 mL of culture was centrifuged for 10 minutes at 7500 rpm. The wet cell mass-containing pellet was washed with sterile distilled water three times and then let dry at 65°C. One-third mL of 2 M HCL was then used to treat 100 mg of dried biomass, then heated in a water bath at 75°C for an hour. Methanol, chloroform, and water were successively added to the acid hydrolyzed biomass in the amounts of 2:2:1.8 (v/v/v). The layer of chloroform comprising all of the lipids from the extraction was obtained by 10 minutes of centrifugation at 8000 rpm with a collection in a pre-weighed vial. The collected chloroform was retained for drying at 65°C until dry. The lipid content is expressed as % w/w of dry cell mass.

Total nitrogen content: The nitrogen content of early stationary and late stationary phase was determined by

the Kjeldahl method (Menefee and Overman, 1940). Two grams of dried biomass sample was weighed accurately and transferred to a Kjeldahl digestion tube followed by the addition of 2 g of digestion mixture comprising of K_2SO_4. $CuSO_4$ and SeO_2 (ratio 100:10:1) and 15 mL concentrated H_2SO_4. The contents were then digested to a transparent clear fluid. The digested material was transferred to a 100 mL volumetric flask, and distilled water was added to make the volume up to the mark. An aliquot (10 mL) of the digest was distilled with 50 per cent sodium hydroxide and the liberated ammonia was collected in 10 mL saturated boric acid containing 2-3 drops of mixed indicator (prepared by dissolving 100 mg methyl red and 30 mg methylene blue in 60 mL of 95 per cent ethyl alcohol and then making up the volume to 100 mL with distilled water). Approximately 65 to 70 mL of distillate was collected in a 100 mL conical flask. The contents of the flask were titrated against 0.02N HCI. A blank analysis using distilled water in place of the sample was also carried out separately, parallel to the main sample. The total nitrogen and per cent protein were calculated as follows:

% Nitrogen content = {14.007(X-Y) × Normality of HCI × dilution factor / 1000 × Weight of samle} × 100

Where, X is the volume of HCl required for the sample, Y is the volume of HCI required for the blank, N is the normality of HCI, and W is the weight of the sample in mg. Crude protein content was also calculated from total nitrogen by multiplying with a conversion factor of 6.38 (Krul, 2019).

Assessment of total carbon and ash content: Yeast biomass was dried overnight at 105°C and subsequently subjected to a temperature gradient with 550°C as the maximum and 105°C as the final temperature prior to cooling in a desiccator. The dry ash weight of the early stationary phase and late stationary phase culture was recorded at 105°C according to standard procedures of the Association of Official Analytical Chemists (AOAC, 1995).

Statistical analysis: All the results obtained from the outcome of this study were presented as the mean of two independent observations along with the SD of the mean. Student-t test was applied to determine the statistical significance of mean data. GraphPad Prizm 5.0 software was used for the graphical representation of data.

RESULTS

Screening of isolates for lipid production: Isolates were screened for the production of lipid by Sudan Black staining protocol. *B. adeninivorans* MTCC 2516 was kept as a positive control during screening. After Sudan black staining of isolates and positive reference culture, the presence of black/blue cytoplasmic granules was checked under the microscope (Fig. 1).

Screening of isolates for *in vitro* safety parameters: Most pathogenic yeasts produce various types of extracellular proteinases to survive and cause infection in the host (Montoya *et al*., 2021). The production of extracellular gelatinase is the one attribute that has been correlated with the pathogenic attribute of infectious yeast strains (Rapala Kozik *et al*., 2018). Mostly oleaginous yeasts being used for bioethanol production are isolates of different environmental samples and are related to various pathogenic species (Abeln and Chuck, 2021). Thereby, it is crucial to check the presence of virulence factors in the oleaginous yeast isolates intended to be used as food grade organisms (Syal and Vohra, 2013). For this, the production of extracellular gelatinase enzyme was checked by allowing the growth of isolates on gelatin agar. After incubation, the colony of yeast culture on gelatin agar was flooded with saturated ammonium sulfate solution. The presence of gelatin hydrolysis would be indicated by a precipitation zone surrounding the colony after ammonium sulphate treatment. The absence of any precipitation zone around our isolates and around the reference strain confirmed negative gelatinase activity.

Haemolytic activity: Hemolytic activity is used to assess the virulence potential of microorganisms. The production of hemolysin is considered a safety-related virulence factor. Many yeast isolates from clinical samples exhibit beta haemolytic activity (formation of a clear halo zone surrounding the colony) on blood agar (Sachin *et al*., 2012; Hussein *et al*., 2019). The absence of a clear zone (beta haemolysis) or green zone (alpha haemolysis) around the colonies of our isolates on blood agar and reference strain indicates none of them possessed any degree of haemolytic activity.

Growth of yeast cultures in whey-based media: The growth pattern of yeast isolates was observed in whey-based media and was compared with the growth of the same strains in standard YEPD broth media. Whey was chosen as a cheaper second-generation media substitute for the commercial YEPD media for yeast biomass production. All three strains maintained the stationary in the range of 65-70 h of incubation period and then showed a decline in viable cell count. The growth curve of all three strains was also analyzed to determine the early and late stationary phases of studied yeast cultures. After careful observation, the period of establishment of early stationary phase and late stationary phase was

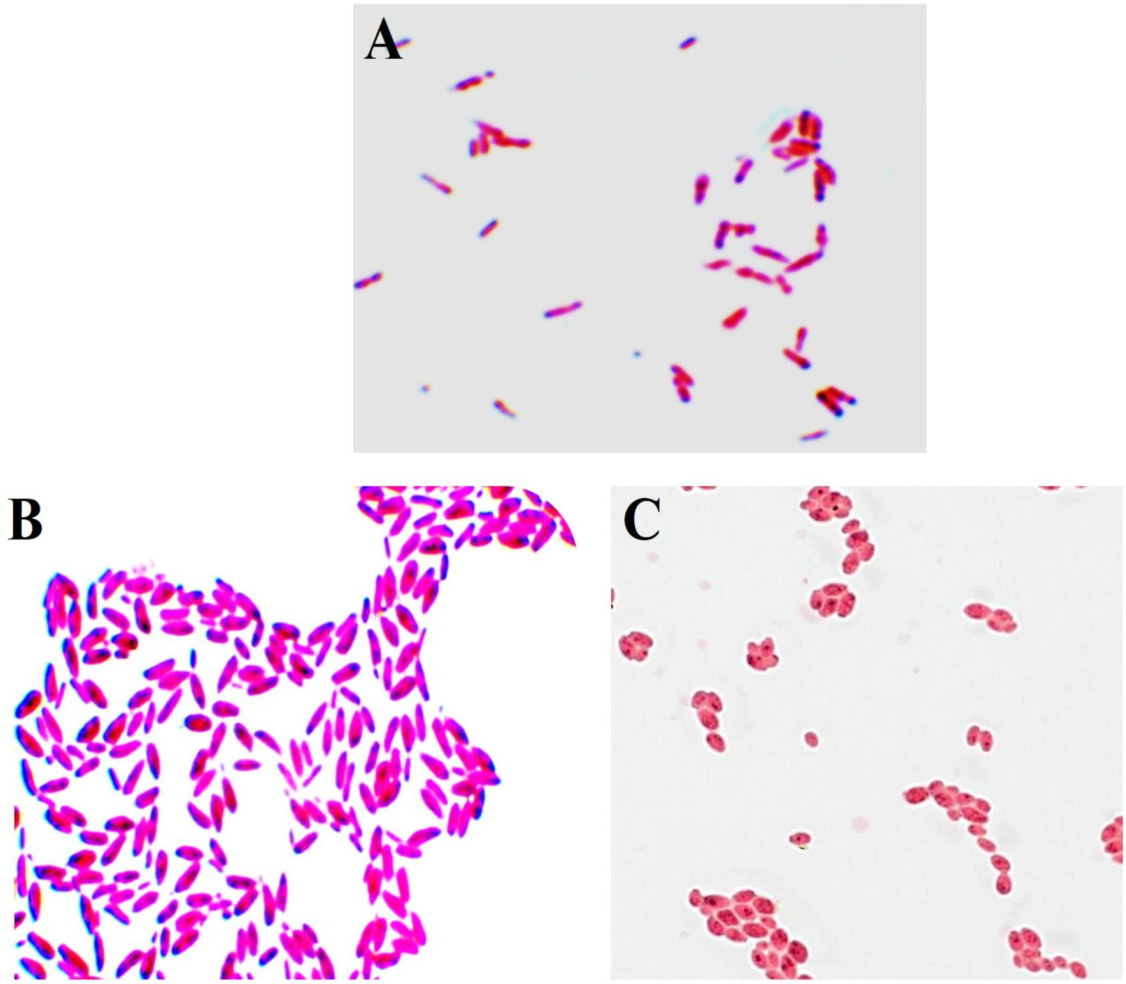

Fig. 1. (A) Presence of black/blue cytoplasmic granules in Sudan black stained cells of *B. adeninivorans* MTCC 2516, (B) CHV (Cheese isolate) and (C) IY (yoghurt isolate)

noted as 24 hours and 68 hours of incubation in batch fermentation system for all three strains. The cultures were able to grow and propagate in whey-based media and showed 1.25 (*Candida zeylanoides* CHV) and 1.34 $\log_{10}$ (*Candida metasilopsis* IY and *B. addeninivorans* MTCC 2516) increases in cell number. In YEPD media the cultures made 1.69, 2.23 and 0.92 $\log_{10}$ increase of CFU/mL for *Candida zeylanoides* CHV, *Candida metasilopsis* IY and *B. addeninivorans* MTCC 2516, respectively.

The biomass of three yeast strains, *B. adeninivorans* MTCC 2516, *Candida zeylanoides* CHV, and *Candida metasilopsis* IY, was quantified during both the early and late stationary phases in whey media. The biomass of *B. adeninivorans* MTCC 2516 was recorded as 0.705±0.005 g/L during the early stationary phase to 0.76±0.04 g/L during the late stationary phase. Similarly, the strain *Candida zeylanoides* CHV exhibited a rise in biomass concentration from 0.7±0.08 g/L to 0.855±0.045 g/L at the late stationary phase. Among all three strains, *Candida metasilopsis* IY had a more substantial increase in biomass from 0.705±0.125 g/L to 0.955±0.265 g/L at the late stationary phase.

Lipid production: The lipid content was extracted from biomass using the organic extraction method and was measured using the gravimetric method. The lipid production from three strains at the early stationary phase was recorded, ranging between 0.54±0.12 g lipid/100 mL whey media *Candida zeylanoides* CHV to 0.57±0.07g lipid/100 mL whey media *Candida metasilopsis* IY. Whereas, at the late stationary phase, 0.53±0.06 g lipid/100 mL whey media (*B. adeninivorans* MTCC 2516) to 0.70±0.22 g lipid/100 mL whey media *Candida metasilopsis* IY. At both the early and late stationary phase, strain *Candida metasilopsis* IY showed maximum lipid production among three tested yeast strains. In comparison to the early and late stationary phase, both of our isolates exhibited an increase in lipid production at the late stationary phase, but the reference strain showed a decline in lipid accumulation at the late stationary phase.

In terms of lipid production from dry cell mass, at the early stationary phase, the maximum production values recorded for *B. adeninivorans* MTCC 2516 (77.92±7.79%) followed by *Candida zeylanoides* CHV (76.17±8.43%). However, the lipid production value given by *Candida metasilopsis* IY (75.79±3.06%) is statistically considered higher due to the lesser standard deviation value. Likewise, in the previous results, strain *Candida metasilopsis* IY showed maximum lipid production among three tested yeast strains at both early and late stationary phase (Fig. 2).

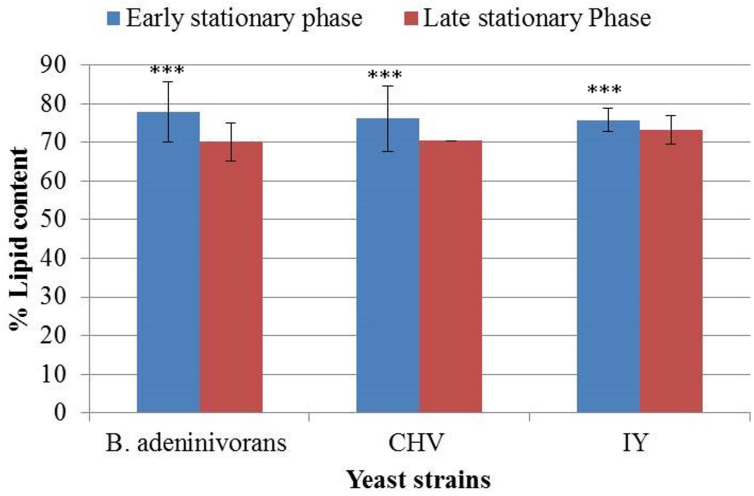

Fig. 2. % Lipid content (W/W of dry cell mass) from the yeast cultures (*Candida zeylanoides* CHV showed significantly higher lipid content (*P<0.001) at the early stationary phase than the late stationary phase)**

Estimation of total protein content: In addition to lipid content, we also analyzed the total nitrogen, carbon and ash content of dry yeast cell mass. The estimation of these four parameters will give us a broad idea of major nutritive components that can be produced by our isolates using second generation feedstock like whey. Firstly, total nitrogen content was estimated from the cell mass collected at the early and late stationary growth phase.

In both phases, our isolate *Candida metasilopsis* IY, followed by *Candida zeylanoides* CHV, exhibited a higher % nitrogen content than the reference strain. This attribute of our isolates may be co-related with efficient whey protein utilization by our isolates. The produced nitrogen content shows a higher level of single-cell protein production in our whey fermentation system, which supplements the value addition of our product. The total nitrogen content is bound to cellular total protein, nucleic acid, nucleopeptides, free nitrogen bases and membrane amino sugars. Besides protein, other components are devoid of nutritive values. Thereby, we further calculated % protein content by multiplying total nitrogen with a conversion factor of 6.38 (Krul, 2019). In accordance with the % nitrogen content of isolates, the % protein content W/W of dry cell mass was produced, ranging

between 43.38±1.72% (*B. adeninivorans* MTCC 2516) to 77.02±3.73% *Candida metasilopsis* IY at early stationary phase. The slight reduction of protein content at the late stationary phase was in the range of 37.76±1.94% (*B. adeninivorans* MTCC 2516) to 59.84±1.03% *Candida metasilopsis* IY. The observations of reduced protein content in the late stationary phase in comparison to the early stationary phase are also reflected here (Fig. 3).

Total carbon content: At the early stationary phase, total carbon content was recorded, ranging between 51.22±2.07% (IY) to 53.45±0.00% (*B. adeninivorans* MTCC 2516). At the Late stationary phase, the same showed an increased range between 53.58±0.07% *Candida metasilopsis* IY to 55.06±0.35% (*B. adeninivorans* MTCC 2516). In both early and stationary phases, *Candida metasilopsis* IY strain exhibited the lowest % total carbon content followed by *Candida zeylanoides* CHV and *B. adeninivorans* MTCC 2516 (Fig. 4).

A combined analysis of % lipid, nitrogen, carbon and ash content (W/W) of dry cell mass of all three strains would give us a clear overview of the nutritive value of the respective strains at their early and late stationary phase of growth. Among three yeast strains, the most efficient conversion of whey media in value added components was done by our yoghurt isolate *Candida metasilopsis* IY. This strain exhibited the highest % lipid content (53% ES, 54% LS) and % carbon content (39% ES, 41% LS) as well as the lowest % ash content (3% ES, 1% LS) of its dry cell mass. However, the relative % protein content was highest in *Candida metasilopsis* IY (75.81% ES, 58.62% LS) (Fig. 5).

DISCUSSION

Our isolated strains showed comparative lipid production compared to the reference strain, which can potentially be used for animal nutrition or food-grade applications as they are food-grade microorganisms. The $\log_{10}$ increase in the growth of oleaginous yeasts in whey media and YEPD were

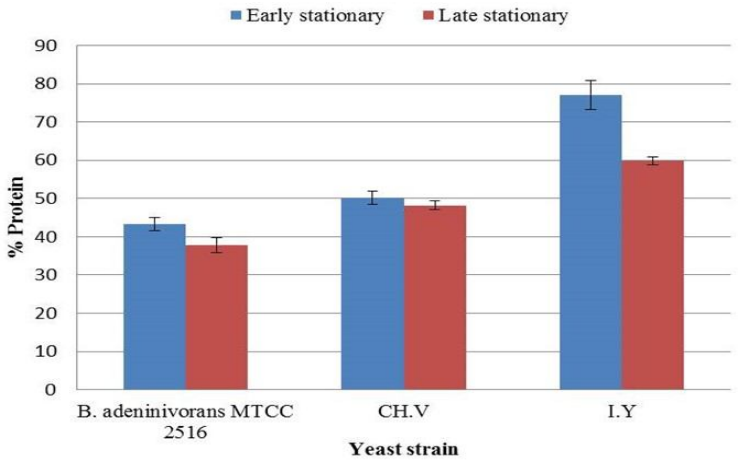

Fig. 3. % Protein content (W/W of dry cell mass) from the yeast cultures (No significant difference (P<0.05) between early and late stationary phase values of cultures)

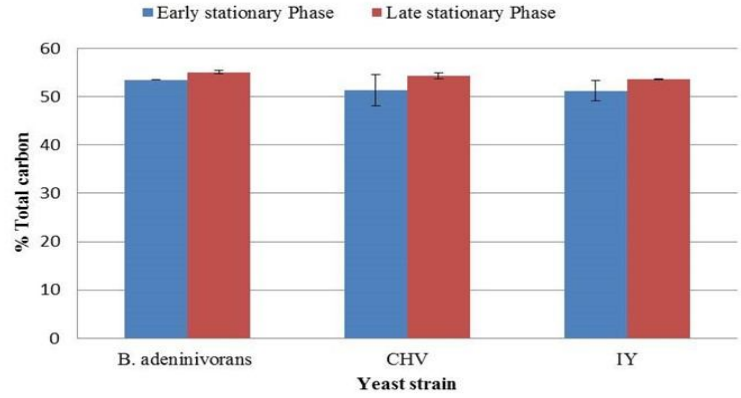

Fig. 4. % Total carbon (W/W of dry cell mass) from the yeast cultures (No significant difference (P<0.05) between the early and late stationary phase values of cultures)

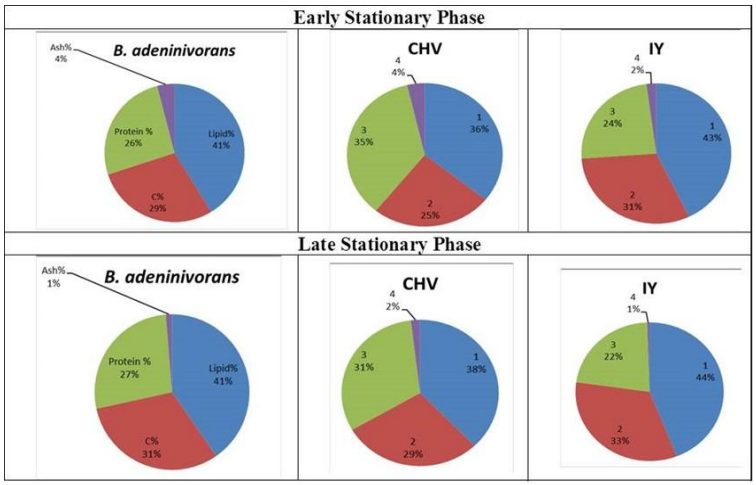

Fig. 5. Pi chart representing comparative analysis of % lipid, protein, carbon and ash content (W/W) of dry cell mass at early and late stationary phase (In figure, 1. represents % lipid content, 2. represents % carbon content, 3. represents % protein content and 4. represents % ash content)

comparable. Various studies in this line have also recorded similar observations during yeast biomass production using whey media. Vamvakaki *et al*. (2010) tested three strains of *Mortierella*, *Thamnidium* and *Mucor* spp. for producing biomass and gamma-linolenic acid using cheese whey. Among three cultures, *Mortierella* sp. showed maximum growth and consumption of a significant amount of lactose and available protein. However, the two other strains were incapable of consuming the lactose after protein was exhausted. The authors were able to achieve higher biomass of both types of organisms by supplementing lactose for *Mortierella* sp. and ammonium sulphate for *Thamnidium* and *Mucor* spp. The added components favoured the growth of cultures as well as increased lipid accumulation in the microorganisms (Vamvakaki *et al*., 2010). Yadav *et al*. (2014) analyzed biomass produced by co-cultivation of *K. marxianus* and *C. krusei* on cheese whey. The study recorded 43.4% of protein, 33.6% of carbohydrates, 8.4% of minerals, 6.4% of lipid, and 4.6% of crude fiber from the tested yeast strains. Another monoculture experiment done by Paul *et al*. (2002) demonstrated protein, crude fibre, ash, and lipid content of *K. fragilis* dry cells cultivated in whey were found to be 4.9%, 16%, and 7.8%, respectively. These previous reports were more focused on the production of food-grade yeast additives using whey substrate. Food-grade yeast extract at a general commercial scale is being used as a source of amino acids, flavouring components, and vitamin supplements in several beverages, bakery items and canned foods. Thus, the production of yeast biomass using whey provides both valuable nutrients with a low environmental footprint.

In terms of lipid percentage, all three strains showed a decrease from the early to the late stationary phase. However, while *Candida zeylanoides* CHV and *B. adeninivorans* MTCC 2516 exhibited relatively minor decreases, *Candida metasilopsis* IY experienced a more notable decline. This suggests a variation in lipid accumulation dynamics among the strains over the growth phases. Regarding carbon percentage, there is a general trend of increase from the early to the late stationary phase for all strains. This could indicate a shift in metabolic processes towards carbon accumulation as cells enter the late stationary phase. Our all tested strains have shown higher lipid production than this. Moreover, a study on *Yarrowia lipolytica* B9 utilized non-sterile semi-defined whey medium for lipid production (Taskin *et al*., 2015). The authors recorded 4.29 g/L lipid production from 7.4 g/L biomass, which made up 58% of the total cell mass. Our observations are also in line with the results recorded by Taskin *et al*. (2015). In another interesting study, Vyas and Chhabra (2019) used *Cystobasidium oligophagum* JRC1 for lipid production using untreated and deproteinized cheese whey. The authors found 0.0335±0.0004 g/L lipid production from 0.076±0.0004 g/L biomass in untreated whey that constructed 44.12±0.84% lipid content of biomass. In a further advanced study, Karim and Aider (2022) produced biomass of *Kluyveromyce smarxianus* ATCC 64884 using through fermentation of electro-activated whey, electro-activated whey-permeate, and electro-activated lactose-based media. The study reported the highest 25.64% and 22.27% lipid content of dry cell weight in electro-activated lactose and electro-activated whey-based media. The researchers in this line have recognized that the recovery of lipid content from such batch fermentation system increases with the increasing biomass accumulation. However, in some cases, the reduction in lipid content at the end of fermentation was also observed (Karim *et al*., 2021). These studies recognized that oleaginous microbes start to accumulate lipid bodies under nutrient-limiting conditions, but after a certain time, the cells may degrade the stored lipids due to the same reason. That's why we studied the lipid production by our tested strains at both early and late stationary phases. The observations and results recorded in this study revealed that the lipid accumulation in our tested yeast strains was stable till the late stationary phase.

Protein percentage showed diverse patterns among the strains. *Candida zeylanoides* CHV and *B. adeninivorans* MTCC 2516 display decrease in protein content from the early to the late stationary phase, whereas *Candida metasilopsis* IY shows a significant increase. This disparity could be attributed to differences in protein synthesis and degradation rates among the strains during the stationary phase. The production of yeasts as single-cell proteins has been explored for a long time. The upcycling of whey protein into yeast single-cell protein in the process of organic load removal it has been reported by several researchers (Yadav *et al*., 2014; Myint *et al*., 2020; Matassa *et al*., 2022). However, the valuation of total protein content from recovered biomass was not the prime object of these studies. Very few reports have stated the analysis of crude protein recovery from whey fermentation using food-grade yeast. For example, Paul *et al*. (2002), grew *Kluyveromyces fragilis* (MTCC 188) in deproteinized whey and observed 37% crude protein content production from dry yeast cell mass.

Overall, the comparative analysis reveals distinct variations in lipid, protein, carbon, and ash content dynamics among the strains during different growth

phases. These differences underscore the importance of strain-specific characteristics and growth conditions in influencing cellular composition and metabolism, which are essential considerations for nutraceutical preparation.

In advance of previous work done in this subject area, we took oleaginous yeast cells in our study for the production of lipid-rich yeast biomass using whey as a substrate. Comparatively, oleaginous yeast has farfetched commercial applications in the biofuel and food market. Their utilization has been explored but not limited as substitutes for cocoa butter, dairy cream, human milk fat, fish oil, bio-lubricants and in drug delivery systems as bioactive lipids (Caporusso *et al*., 2021; Bhutada *et al*., 2022). The establishment of an economically feasible high-yielding food grade oleaginous yeast culture system is being explored by researchers nowadays. Our work, in this line, will also provide an important commencement for the development of an economical and environment-friendly method to achieve the production of high-value products by the direct utilization of whey. The two yeast isolates are safe to use as single cell oil and single cell protein in human nutraceutical formulation and animal feed supplementation.

Conflict of interest: The authors have declared that no conflict of interest exists in the study.

Author's contribution: DD: Performed the lab work and data compilation; NR: Designed the study and prepared the draft; SKM, NG, DK: Helped in research work and preparation of the manuscript.

Data availability statement: All the data supporting the research findings have been presented in this paper. The corresponding author is willing to provide the raw data upon reasonable request.

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