D2.5 – Report on ABC- BioPhosphate: multi-functional biofertiliser and adsorbent

WP2, T2.4 ABC-BioPhosphate: multi-functional biofertiliser and adsorbent

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Executive Summary

The objective is to fully upcycle an unexploited dairy waste water (WW) stream acidic whey, which is consisting of 93% water, and presents extremely high BOD/COD/TOC and as low pH as 4.5. Acidic whey is a problematic liquid produced in the overwhelmingly large quantities. WW by-product stream of the dairy food industry with significant economic importance. This problematic liquid stream is converted into P/Ca based multi-functional compound biofertiliser with combined effects by multi-stage processing for a wide range of product functional applications (as of safety, quality and labelling compliance defined by the (EU) 2019/1009 PFC categories) and high nutrient recovery efficiency while environmental impacts minimised. The liquid fermenter will be inoculated by a selected agriculturally beneficial microbial strain (*Trichoderma harzianum*) that is specifically selected for the acidic whey biotech processing. In this context, the problematic WW is transformed into resource and converted into market competitive multifunctional biofertiliser. The sustainable reuse of acidic whey closes the nutrient loop for the dairy farmers and food processing industry. The high nutrient density compound Bio Based Fertiliser (BBF) BIO-NPK-C output products delivered to regional users, who will use it for their onsite CMC3 green compost material enrichment and improvement where the technological irrigation water effluent is also reused.

The reduction of pollution, energy use and greenhouse gas emissions, aiming to fully close the dairy industry nutrient cycle for dairy food processing where energy- and climate neutrality are in focus. Economically viable full circularity is targeted to be implemented towards high Technology Readiness Level (TRL) and Business Readiness Level (BRL).

The biotech fermentation upcycling process is considered as the main treatment line. The adsorption processing is a support to complete and close the nutrient and water cycle loops for the dairy farmers and processors, while reducing energy consumption and environmental/climate impacts. Beyond the main target of the dairy WW acidic whey stream processing, other types of food industrial WW processing possibilities also considered for possible application of suitable for WalNUT added value nutrient recovery new business model.

Animal Bone Char (ABC) is highly optimal and efficient to adsorb macromolecular organic contamination in liquid stream and adjust pH. The ABC animal bone char adsorbent is recently REACH registered and certified by the EU ECHA Authority (1-10 t/y) that is the regulatory precondition for lawful TRL5 implementation for this case. The combination of fungus biotech fermentation main process with adsorption processing is suitable for WalNUT added value new nutrient recovery business model with “Blue Ocean” Strategy.

The reuse of effluent water for moisture control of cattle manure, co-compost, and close the nutrient cycle is a viable solution for efficient upcycling of the unexploited dairy industrial WW acidic whey. The full circularity and zero emission targets are also implemented.

Keywords: **Keywords**: dairy industry, acidic whey, upcycling, nutrient recovery, circular, zero emission, Animal Bone Char, BioPhosphate, food industrial waste water, REACH, close nutrient cycle.

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Abbreviations

ABC	Animal Bone Biochar - BioPhosphate
AW	Untreated acidic whey
ref %:	soluble solids determined by the refractometer
% V/V	volume/volume percent
WW	Waste Water
BOD	Biochemical Oxygen Demand
COD	Chemical Oxygen Demand
TOC	Total Organic Carbon
REACH	European Union regulation for Registration, Evaluation, Authorisation and Restriction of Chemicals
ECHA	European Chemicals Agency
TRL5	Technology Readiness Level 5 (product is defined, technology and lawful application is validated in relevant environment with large scale prototype)
BRL5	Business Readiness Level 5 (a deep understanding of the targeted market application for the defined product is achieved and the value proposition is validated)
Upcycling	High added value transformation of unexploited biomass into new products, perceived to be of greater quality and environmental/climate value with second life and new function that finished product becomes more practical and valuable than what it previously was.
"Blue Ocean" Strategy	The "Blue Ocean" Strategy open new market space and create new demand that is creating and capturing uncontested market space.
Zero emission	The zero emission means that all material streams in all forms and energy flows are processed and converted into useful products.
BBF	Bio Based Fertiliser
Close the dairy industry nutrient and water cycle loops	Closing the dairy industry nutrient cycle for carbon, nitrogen, phosphorus and water is a process which aims to compensate the exit of mineral elements that are necessary for animals and plants that is an essential contribution to the circular economy. Closing those cycles improving self-sufficiency of dairy farms. Tackling the existing nutrient flow gaps in Europe will help decrease greenhouse gas emissions, reduce soil degradation and improve EU independence for energy, nutrients and water supply to dairy farm operations, such as irrigation water to moist on-farm composting.
PTEs	Potentially Toxic Elements
SSFF	Solid State Fermentation and Formulation
CFU	Colony Forming Units

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1. Introduction

1.1. Purpose, scope and target group

The objective of the 3R is to develop, design and test integrated biological (fermentation, biomass production) and adsorption technology for food industrial WW streams with variating contaminations for low-cost production of recovered BBFs and clean irrigation water to moisturise manure and green composting at dairy farm operations. The biotech fermentation upcycling process is considered as the main treatment line and the adsorption processing is a support to complete and close the nutrient and water cycle loops for the dairy farmers and processors, while reducing energy consumption and environmental/climate impacts.

The 3R objective is the integrated upcycling of food industrial WWs (main target: acidic whey) and by-products to improve agri resource management for less cost. The 3R develop, design and test a specific liquid and solid-state fermentation technology with interconnected adsorber where the food industrial WW will be used as a liquid medium during the fermentation process. If required additional food industrial by-products will also be added to the liquid medium during the fermentation. The liquid fermenter will be inoculated by a selected agriculturally beneficial microbial strain (*Trichoderma* spp).

The 3R liquid fermentation system will eliminate the phytotoxic compounds from the food industrial WW and at the same time, high concentration biomass of the agriculturally beneficial microbial strain will be produced. During the fermentation process, the organic compounds of the WWs and by-products are converted into safe and added value recovered and upgraded microbial biomass. After finishing the liquid fermentation, the biomass will be separated from the liquid phase. The obtained microbial biomass will be used to inoculate the solid fermentation system where Animal Bone Char (ABC) carrier is added. The end-product is P/Ca enriched BBF in economical nutrient density and microbial plant biostimulant. The final product quality and safety will be checked.

3R will also develop, eco-design and test a new adsorption technique to apply a special and macroporous adsorbent ABC with specific physico-chemical character, that is a food industrial by-product recovered organic economical fertiliser itself. ABC adsorbent will be used for the final treatment of the separated liquid effluent from the liquid fermentation process. The end-product is clean irrigation water for horticultural applications or other industrial uses. The final product quality and safety will be checked.

The purpose of Task 2.4. is the concept development and laboratory scale test of the ABC-BioPhosphate: multi-functional biofertiliser as the main treatment line, which is supported by adsorbent technology. Concept development of the liquid/solid state fermentation and integrated ABC-BioPhosphate adsorption technology of dairy food industrial WW stream (acidic whey) with variating contaminations for low-cost production of recovered BBFs and to obtain a high quality final effluent upcycled clean water. Laboratory scale validation of the fermentation at 3x3 L scale made.

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1.2. Contribution partners

Table 1: Contribution of partners

Partner	Contribution
3R	Concept development and laboratory scale test of the ABC-BioPhosphate: multi-functional biofertiliser and adsorbent technology.

1.3. Relation to other activities in the project

Table 2: Relation to other activities in the project

Task	Description
Task	**Description**
1.1.	Inventory nutrient database and nutrient imbalances analysis related to dairy food industrial WW processing.
1.2.	European status and barriers on nutrient recovery technologies and bio-based fertilisers related to dairy food industrial WW processing.
1.3.	Web-based knowledge platform for agricultural nutrients from secondary resources recovery related to dairy food industrial WW processing.
2.1.	Harmonised protocol for the characterisation of waste water streams and validation technologies at lab scale related to dairy food industrial WW
2.4.	Concept development of the liquid/solid state fermentation and integrated ABC-BioPhosphate adsorption technology of dairy food industrial WW stream acidic whey with ABC-BioPhosphate multi-functional biofertiliser and adsorbent. Characterise, design and test dairy industrial acidic whey WW upcycling technology for NR at lab scale, while considering technical, economic and environmental aspects. D2.5.
2.7	WalLAB methodology to scale-up technologies to pilot level related to dairy food industrial WW
3.1.	Standards for integration technologies into pilot plant related to dairy food industrial WW.
3.1.1.	Characterisation of dairy food industrial WW stream acidic whey.
3.1.2.	Effluent characterisation of dairy food industrial WW stream acidic whey pilot and assessment for it potential usage for different purposes as reclaimed water (irrigation, cleaning) according to the legislation.
3.1.3.	Process of design, construction and installation CAD submission for pre-operation follow up
3.4.	Design, construction, installation and operation of the TRL5 Pilot plant 3. M14-M18 design 3R TRL5 pilot plant. M19-M24 build 3R TRL5 pilot plant. M25-M26 start-up 3R TRL5 pilot plant. M27-M38 operate and optimise 3R TRL5 pilot plant to produce enough BBFs to perform field trial. Lawful validation of TR5 pilot for REACH 1-10 t/y and legal certification to be completed before M18. Multi-functionality safety, quality and labelling compliance as of (EU) 2019/1009 PFC categories to be completed. P1-Product: Fungus formulated ABC BBF. P2-Product: Nutrient formulated multifunctional adsorbent ABC BBF.
4.4.3	Field validation of agronomic performance in quadruplicate-randomised block design utilisation. M12-M18 assess quality requirement and EU regulation conformity, including REACH. M19-M24 assess and prepare

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	demo action. M25-M54 demonstration and validation of BBF at relevant scale and identify the application practices
4.5.	REACH relevant eco-toxicological testing of WW derived innovative BBFs utilisation. REACH guidance for sector between 1 and <10 t/year.
5.1.	Development and implementation of data compilation strategy, literature review related to the dairy food industrial WW stream acidic whey.
5.2.	Inventory analysis, modelling environmental emissions and soil fertility improvements and upscaling of technology performances review related to the dairy food industrial WW stream acidic whey.
6.1.	Inventory and integrated assessment of the EU regulations related to production and application of bio-based fertilisers.
6.2	Policy recommendations for regulatory, market and user acceptance of BBFs from WW.
6.3.	Short and medium and long term impact assessment of the results for policy, markets, environment and climate in EU dimension. Policy implications at regional/national and European level (Bring knowledge from WP1-5. collaborates with WP7, WP8)
7.1.	Identification of business opportunities within full value chain related to the dairy food industrial WW stream acidic whey upcycling results.
7.2	WalNUT circular WWT business model related to the dairy food industrial WW stream acidic whey upcycling results.
7.3.	Exploitation plan related to the dairy food industrial WW stream acidic whey upcycling results.
8.1.	Communication, Engagement, Dissemination strategy and Community Management related to the dairy food industrial WW stream acidic whey upcycling results.
8.3	Public communication.
8.4	Stakeholder relations, multi actor approach and engagement related to the dairy food industrial WW stream acidic whey upcycling results.

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2. State of art

2.1. The dairy industrial challenges

The livestock production sector in the European Union (EU) is extensive and constantly expanding. Milk production takes place in all EU countries and represents a significant proportion of the value of EU agricultural output. As of EU Agriculture and Rural Development milk market observatory the total EU milk production is estimated to be around 155 million tonnes per year from which 9.1 million t/year are processed for cheese and 7.3 million t/year for fermented milk resulting in large volumes of whey WW. Food chain wastes notably dairy by-products and WW sewage sludge are important and promising sources for the recovery and reuse of nutrients such as P and N (Buckwell and Nadeu, 2016; EIP-AGRI, 2017; Campos et al., 2019). The need to sustainably reconnect nutrient flows between plant production and these waste streams or sources and invest in agro-industrial processes is gaining increasing attention (Scholz et al., 2014; Withers et al., 2018; Powers et al., 2019; Akram et al., 2019; Drangert, 2020). This is supported at the EU level by a number of key strategies and legislations. The EU Waste Framework Directive (2008/98/EC) and Amending Directive (2018/851) broadly establish a legal framework for treating waste using recovery and recycling techniques to reduce pressure on resources and harness valuable nutrients from waste streams. This is elaborated in the EU Fertilising Products Regulation, Circular Economy Action Plan as well as in the 'Farm to Fork' strategy within the EU Green Deal, which emphasises the need to reduce agricultural losses of nutrients and impacts on surface and groundwaters. The advantages of closing the loop on the nutrient cycle range from less dependency on imported fertilisers (Powers et al., 2019) to the creation of employment across the value chain - processing, transportation, marketing, and distribution of recycled products (EIP-AGRI, 2017) for use as fertiliser, fuel, and feedstock. Despite the potentials that recovery and reuse of nutrients from food industrial and agricultural WW streams offer; several technical, institutional, social, economic, and environmental constraints remain to be addressed. A comprehensive consideration of these constraints is important in determining the appropriateness, effectiveness, and sustainability of processes and technologies in recovering nutrients, mitigating contaminants, improving food safety, and enhancing soil health and function (Bernal, 2017; De Vrieze et al., 2019). The specific challenge of the dairy industrial acidic whey WW is the high BOD/COD/TOC at low 4.5 pH.

2.2. The problem of the food industrial origin acidic whey waste water

Acidic whey is a dairy food industrial waste water stream with 93% water content and low pH. This large volume effluent arises during the macroscale production of traditional cottage cheese and similar products that have significant economic and EU food market importance. Whey is the central side stream in the dairy sector. Whey is the liquid remaining after milk has been curdled and strained. It is a by-product of the manufacture of cheese, cottage cheese or Greek yogurt or casein (A Bosso *et al.*, 2020).

Worldwide cheese whey production by dairy industry is estimated at $190 \cdot 10^6$ t/year being resulted of production of cheese or to remove casein from milk and which cause serious economical and environment problems. (A Bosso *et al.*, 2020).

Cottage cheese (traditional lumpy curd cheese) is made by traditional production method. This dairy product is created with milk coagulation using lactic acid bacteria. Lumpy curd cheese is formed when lactic acid organisms produce enough acid from the fermentation of lactose to cause coagulation of casein in skinmilk. The raw milk inoculated by lactic acid bacteria culture. The bacteria culture is acidifying milk, which results in the curds. During the curd production process, the water content is regulated to reach the required level by first-pressing, heating and second pressing. The next step in the curd processing is cooling and draining (whey separation). The draining removes the acidic whey from the curd. (Hungarian Milk Interprofessional Organization and Product Board, 2022)

For every 100 kg milk used in Greek yoghurt production, only one third ends up in the final product, while the other two thirds become acid whey. Both sweet whey and acid whey are composed mainly of water (93%) while the solid components consist of minerals (12–15%), lactose (70–72%) and whey proteins (8–10%). The

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largest difference between acid whey and sweet whey is the pH, which lies within 6.0–6.5 for sweet whey and 3.6–4.5 for acid whey. Acid whey has less protein, is more acidic and has a more distinct (sour) taste. (Bolwing *et al.*, 2019).

The utilisation of acidic whey is limited due to its different composition from sweet whey. The challenges of utilising acid whey occur in the processing procedure. The most common way to process whey into a product suitable for industrial use is to dry it through evaporation in multistage vacuum evaporators followed by spray-drying. However, spray-drying acid whey with conventional technology is not feasible due to the high content of lactic acid, which makes the whey powder more likely to absorb moisture, resulting in an increased stickiness of the powder. Moreover, a low pH makes the proteins less stable, and it is more difficult, for instance, to remove water from acid whey than from sweet whey. Because of the low pH and the proximity to the isoelectric point, the protein will readily precipitate, which may make it difficult to recover. Proteins are thus more readily available and easily isolated from sweet whey than from acid whey. Moreover, sweet whey can easily be heat- treated and used in new products (e.g., creamy cheese). If acid whey is heated, it will not become an acidic gel, but unites and acquires a slightly granular consistency. Although acid whey is a suitable feed, the transport and storage of liquid whey is costly and barely profitable. (Bolwing *et al.*, 2019).

Acidic whey is considered to cause environmental problems, disrupting the natural balance of rivers and streams and currently companies often pay for it to be drained or dumped across farmers' fields. Acidic whey is comprised of 93% water, it remains the other properties of acid whey such as lactose, lactic acid, calcium, phosphorus and galactose that create issues when trying to turn it into more than just a by-product of Greek yoghurt of cream cheese (Bolwing *et al.*, 2019).

Acid whey is a potential hazard to the aquatic environment due to its high organic matter content in the shape of lactose, resulting in a high Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD). This makes waste water treatment expensive, particularly in the case of small and medium sized milk processing plants. (Bolwing et. al 2019).

The high BOD level means that the presence of acid whey in waters would cause a drop in biological oxygen levels, leading to the elimination of aquatic life. Hence, if other uses cannot be found, acid whey must be treated as waste water in own or municipal plants, involving significant financial costs for the dairy as well as socio-economic costs associated with waste treatment. (Bolwing *et al.*, 2019).

If acidic whey is not processed, it quickly turns sour due to the formation of lactic acid. If its utilisation cannot be resolved, the large amount of acid whey must be separated from the other waste water, because it would result in an unacceptably low pH value for on-site waste water treatment or discharge into the public sewer system.

2.3. Potential agricultural use of *Trichoderma harzianum* fungus as a microbiological substance

Trichoderma species are among the most distributed fungi in nature and typically they are found in agricultural and forest soils in a wide range of eco-systems and climatic conditions from continental, Mediterranean, tundra as well as tropical soils. Their ability to survive in different regions can be attributed to diversified metabolic capabilities, natural competitive aggression (Nur *et al.*, 2020), (Lopes *et al.*, 2012) and high capability for degradation of cellulose/remaining plant biomass in the soil. *Trichoderma* is a filamentous fungus that is one of the most widely studied genera with many applications in agriculture and the environment. (Joo *et al.*, 2022).

A significant advantage of *Trichoderma harzianum* is that they are growing on a wide range of carbon and nitrogen sources and can be induced to produce hyphal biomass, conidia, or chlamydospores suitable for a variety of applications including the production of biofertiliser.

Trichoderma strains can be identified by common morphology characteristics which are a bright green conidial pigment, rapid growth and repetitively branched (Nur *et al.*, 2020).

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Trichoderma spp. possess a wide variety of mechanisms for survival and proliferation, including physical attack and degradation of other fungi, and utilisation of complex carbohydrates. Based on these characteristics, *Trichoderma* spp. are of considerable economic importance, and are used in commercial applications such as industrial enzyme production, heterologous protein expression, antibiotic production, and biocontrol of plant pathogenic fungi (Lopes *et al*., 2012).

Trichoderma seems to be the best candidate for use in green technologies due to its wide biofertilisation, nutrient mobilisation and biostimulatory potential which are the prime mode of action for this substance. Most of the species from the genus *Trichoderma* belong to the plant growth-promoting fungi that produce phytohormones and the 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme. *Trichoderma* improves overall plant health, by creating a favourable environment and production of a large number of secondary metabolites that can stimulate plant growth such as auxins, ethylene and other volatile organic compounds. *Trichoderma* strains are is directly giving an impact on plant development and crop productivity by promoting plant health (Harman *et al.,* 2004).

Trichoderma is an excellent plant growth-promoting fungi. Development of root is the common benefit of *Trichoderma* for plant growth as produces or controls of plant hormones that are responsible for improving root development such as auxin, harzianic acid and harzionalide by *Trichoderma* spp. Rhizosphere filamentous fungi of the genus *Trichoderma*, a dominant component of various soil ecosystem mycobiomes, are characterised by the ability to colonise plant root.

They have been shown to promote plant growth via increased nutrient uptake and to protect them against biotic and abiotic stresses. When propagative *Trichoderma* structures, such as spores, are added to soil, they come into contact with plant roots and can germinate and grow on root surfaces, and some *Trichoderma* strains can infect the outer few root cells. *Trichoderma* spp. produce at least three classes of compounds that elicit plant defence responses: peptides, proteins and low-molecular-weight compounds. Root colonisation by *Trichoderma* spp. also frequently enhances root growth and development and can therefore improve crop productivity. *Trichoderma* spp. also increase nutrient uptake and the efficiency of nitrogen use and can solubilise nutrients in the soil. (Harman *et al.,* 2004).

Trichoderma produces organic acids and chelating metabolites such as oxalic acid, citric acid, dl-malic acid, succinic acid, dl-lactic acid, and fumaric acid and has the ability to solubilise many plant nutrients from their solid-phase compounds (Altomare *et al.,* 1999). They are suitable for the breakdown of different organic matters in the soil such as cellulose. *Trichoderma harzianum* produces cellulose-degrading enzymes such as exoglucanase (EXG), endoglucanase (EG) and β -glucosidase (BGL) degradating the remaining plant biomass in the soil. Nutrient cycling can be achieved by the role played by decomposer fungi such as *Trichoderma* in returning nutrients from dead organic matter back into the soil. *Trichoderma* is a good natural decomposition agent that enhances the rate of organic matters decomposition process and therefore organic nutrient cycling. Many researches shows the production of compost inoculated with *Trichoderma* spp. successfully improve the soil nutrients and increase the growth of crops (Nur *et al.*, 2020).

Amongst all the plant nutrients, phosphorus (P) is probably present in the soil in the forms with the most limited bioavailability to plants. The soil application of *Trichoderma* strains was demonstrated experimentally to increase inorganic phosphate solubilisation due to extra-cellular phytase activity (Saravanakumar, *et al.*, 2013) and acidification of the soil environment by acetic, butyric, citric, and fumaric acids production (Scervino *et al.,* 2013).

A special benefit is the induction of increased nitrogen use efficiency (NUE) in plants. This mechanism is not enlightened yet but probably is connected to *Trichoderma* stimulation of deeper rooting, and thereby increasing the volume of soil colonised by plant roots. Sucrose metabolism increased by *Trichoderma* stimulates the resistance response in the leaves leading to increased photosynthesis and respiration because growth induced by *Trichoderma* plant requires energy, and sunlight energy utilised in increased photosynthesis will be the energy source needed for plant growth enhancement by *Trichoderma* mechanisms. (Saravanakumar, *et al.*, 2013)

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2.4. The aim of the adsorption technology task applied for dairy industrial acidic whey waste water processing

The aim of the innovative adsorption technology task applied is to remove macromolecular organic pollutants from dairy industrial WW efficiently by application of unique macroporous adsorber Animal Bone Char (ABC). ABC is highly suitable as bio-carrier for soil biotech formulations, soil applications and low pH substance processing, and contains economically high phosphorus density as well. The objective of the adsorption tests is to process the liquid phase of the fermentation biotech line, make direct treatment for the food industrial WW acidic whey fertiliser line and produce technological irrigation clean water to be used for moisture control of the co-composting main application. Two types of acidic whey liquid streams processed by adsorption, such as:

1) Direct adsorption processing of the dairy WW stream acidic whey and
2) Adsorption processing of the separated fermentation liquid residuals.

2.5. State of art of the 3R adsorption technology applied for dairy industrial acidic whey waste water processing

Activated carbon is a material that is widely used in industries as an adsorbent and purifying material. However, different specific applications require specific types of adsorbents. In the WalNUT case dairy food industrial WW acidic whey recovery and upcycling solution targeted in line with the objectives of the EIP on Agrifood and Raw Materials aiming biofertiliser production. In this context, only bio-based adsorbents counted, such as non-activated – natural ABC and coconut-based activated carbon.

Activated carbons, usually made from coal or coconut shell basic materials, are widely used as commercial adsorbents to remove specific target compounds or classes of compounds from waters and waste waters containing complex mixes of substances. A variety of standards and standardised methods are available to determine physical, physico-chemical, surface-chemical, structural, and adsorptive properties that have been standardised by the American Society for Testing and Materials. The inner surface, the total pore volume, the micro- and mesopore volume as well as the pore size distribution are important parameters. The pore size provides important information such as whether adsorptive molecules can penetrate the pore system and be adsorbed, the size of the surface and how the pore volume influences the maximum number of molecules that can be adsorbed. In order to ensure an effective adsorption process and effects, specific adsorbents must be selected for each treatment case.

Coal and coconut shell adsorbent activated carbons are microporous with pore size <2 nm and mesoporous with pore size 2 – 50 nm. The specific macroporous adsorbents have pore size above 50 nm. A unique product of this category of adsorbents is the ABC, which is a biobased apatite – calcium phosphate mineral. Activated carbon is a material that is widely used in industries as an adsorbent and purifying material. However, different applications require different types of adsorbents. In the WalNUT case dairy food industrial WW acidic whey recovery and upcycling solution targeted in line with the objectives of the EIP on Agrifood and Raw Materials aiming biofertiliser production. In this context, only bio-based adsorbents counted, such as non-activated – natural ABC and coconut based activated carbon.

While traditional bone char products have been known since 1820 in the UK, and since 1870 in large industrial scale produced in the UK, the operations stopped in 2001 as the outdated technology did not meet the new EU industrial/environmental regulations and quality requirements anymore and the old system could not be further developed either. The WalNUT project is innovative upgrading and widening the application opportunity of the modernised ABC for specific food industrial WW acidic whey processing to recover multifunctional biofertiliser products.

ABC is an innovative adsorber which can be thermally regenerated as coconut shell activated carbon adsorber. The low pH is adjusted and neutralised by the ABC high Ca content in combination with food grade sodium bicarbonate powder water soluble additive (sodium hydrogen carbonate CAS 144-33-8 at pH 8.3). The high BOD, COD and TOC are decreased to meet the permissible limits. However, during the thermal regeneration of the spent adsorber the biofertiliser component (that is the main WalNUT concept) is lost.

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The multifunctional ABC adsorbent used for the recovery and upcycling of the acidic whey dairy food industrial WW. The solution is consisting of two applications for which the final product quality and safety will be checked:

a) Solid microbiological carrier for fungus adaptations during solid-state fermentation, which biofertiliser also contains economically high P nutrient density (35% P_2O_5).
b) Final treatment of the separated liquid effluent from liquid fermentation process. The end-product is clean irrigation water.

The surface area, a unique macroporous structure, alkaline character and a high degree of surface reactivity make the ABC versatile adsorbent in the case of food industrial WW acidic whey. The physico-chemical characteristics of the adsorbent alternatives evaluated to justify that the optimum adsorbent for the WalNUT case is ABC are summarised in Table 2-1:

Table 2-1: Comparison of ABC Animal Bone char and coconut shell based activated carbon

ADSORBENTS	Alternative A ABC Animal Bone Char (food grade animal bone based)	Alternative B Activated Carbon (coconut shell based)
Material composition	92% calcium phosphate bioapatite mineral 8% carbon	91% carbon 9% ash
Spec. surface area	80 - 120 m^2/g BET	500 - 1200 m^2/g BET
Iodine	100 – 300 mg/kg	800 – 1200 mg/kg
Ash	1% max.	5% - 9 %
Hardness	98%	98%
Porosity	40-63,000 nm macroporous 100%	2-20 nm mesoporous 10%, microporous 90%
Pore volume	0.55 cm3/g	0.49 cm3/g
Bulk density	650 kg/m3	500 kg/m3
Mesh size	4x8, 6x12, 8x20,8x30, 12x40, 20x140	4x8, 6x12, 8x20,8x30, 12x40
pH	8 – 10.5	7-11.5
PTEs mg/kg	Cd <0.3; CrVI nd; Hg 0.03; Ni 2; Pb 1; As <1; Cu 13; Zn 89.	Cd 1; CrVI nd; Hg 0.5; Ni 10; Pb 50; As 20; Cu 50; Zn 10.
PAHs	1 mg/kg (PAH 19 as of MS regulation)	4-6 mg/kg (PAH 16 as of EU regulation)
Phosphorus	155,000 mg/kg (35.5% P_2O_5)	2,000 mg/kg
Calcium	259,000 mg/kg (36.2% CaO)	50,000 mg/kg
Application areas	High efficiently remove macromolecular organic pollutants such as from food industrial WW. Highly suitable as bio-carrier for soil biotech formulation, soil applications and low pH substance processing. Having unique macroporous structure and surface characteristics. Containing economically high phosphorus density.	Remove nano and micro chemical pollutants, water purification, gas adsorber, pharmaceutical and chemical industries, solvent adsorption. Not suitable for soil applications and cannot be bio-formulated.
Processing temperature	850 °C material core	1000 °C material core and activation
Energy consumption	Energy self-sustaining and surplus energy producer	Highly energy demanding and intensive.
Reagent consumption	No reagents or chemicals used. Not activated, natural char.	High toxic chemicals used for activation, chemical activation with potassium hydroxide (or NaOH or H_3PO_4 or $ZnCl_2$)

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Space requirements	Medium scale industry space	Medium scale industry space
Distance (from the source) /Transport impact	Regional short supply chain for the European origin input feed material	Transcontinental long supply chain for the tropical origin input feed material
Carbon footprint	Zero emission processing and energy independent	Highly negative environmental/climatic impacts
Adsorbent regeneration	Yes, 3-4 times, thereafter reused as soil biofertiliser.	Yes, 3-4 times, thereafter incinerated.
Market price	1500 €/t	4500 €/t

The appropriate method with appropriate precision, reproducibility, detection limits and recovery was designed for the adsorption tests. The results were practically applicable at TRL5 large scale prototype design that is applied in relevant environment. Adsorptions tests and immersion experiments were made to measure adsorption equilibrium and kinetics from solutions. The tests consist of the addition of a known mass of sample to a fixed volume of liquid at an initial concentration. The adsorption amount is calculated by subtracting the number of remaining molecules at the adsorption equilibrium from the number of introduced molecules.

2.6. The novelty of the new specific adsorber for processing of acidic whey food industrial waste water

Although animal bone char materials known since early 19th century, the traditional bone pyrolysis and products did not meet the 21st century regulations. Therefore, under the EU RTD framework programs new innovative solutions are developed for high temperature carbonisation of animal bones and make advanced bone char products (Someus *et al.,* 2018).
The application of the multifunctional ABC novel adsorbent in the dairy industrial acidic whey waste water processing is a novel approach. The unique characteristics of the innovative adsorber are:

1) Full macro porosity,
2) Ability to adsorb macromolecular organic contaminants efficiently,
3) High P content, economical high nutrient density,
4) High Ca content to neutralise the low pH acidic whey, and
5) Regeneration and reuse options.

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3. Material and methods

3.1. Analytical characterisation of the acidic whey waste water feedstock

The analytical parameters were measured by external accredited laboratory, Balint Analitika Ltd. The sampling made by 3R. The following Table 4-1 lists the test methods.

Table 3-1: Test methods for measurement of analytical parameters of acidic whey

Parameters	Test methods
pH measurement	SZ 260-4:1971 Chapter 3 Measuring range 1-13pH units. Measurement uncertainty: ± 0.05 pH units
Specific electrical conductivity measurement	MSZ EN 2788:1998. Measurement uncertainty: ± 25% Lower measurement limit: 10µS/cm
Determination of chemical oxygen demand (COD_5)	MSZ ISO 6060:1991 Measurement uncertainty: ± 25% Lower measurement limit: 30 mg/L
Determination of biochemical oxygen demand (BOI_5)	MSZE 21420-9:2004 Chapter 9 Measurement uncertainty: ± 25% Lower measurement limit: 3 mg/L
Determination of organic nitrogen content (calculation)	MSZ 260-12:1987 period6.2. Measurement uncertainty: ± 25% Lower measurement limit: 0.5 N mg/L
Determination of nitrogen content (calculation)	NSZ 260-12:1987 period6.3. Measurement uncertainty: ± 25% Lower measurement limit: 0.5 N mg/L
Determination of ammonium and ammonium-N content	MSZ ISO 7150-1:1992 Measurement uncertainty: ± 25% Lower measurement limit for ammonium:0.01 mg/L Lower measurement limit for ammonium-N: 0.008 mg/L (calculation)
Determination of nitrate and nitrate-N content	MSZ 260-11:1971 Measurement uncertainty: ± 25% Lower measurement limit for nitrate: 0.3 mg/L Lower measurement limit for nitrate-N: 0.07 mg/L (calculation)
Determination of nitrite and nitrite-N content	MSZ 260-10:1985 Measurement uncertainty: ± 25% Lower measurement limit for nitrite: 0.01 mg/L Lower measurement limit for nitrite-N: 0.003 mg/L (calculation)
Determination of suspended matter content	MSZ 260-3:1973 Chapter 5 Measurement uncertainty: ± 25% Lower measurement limit:2 mg/L
Determination of total phosphorus content	MSZ 260-20:1980 Measurement uncertainty: ± 25% Lower measurement limit: 0.02 P mg/L
Determination of sulphate content	MSZ 260-7:1987 Chapter 2 Measurement uncertainty: ± 25% Lower measurement limit: 10 mg/L
Determination of ortho-phosphate-P content	MSZ EN ISO 6878:2004 Chapter 4 Measurement uncertainty: ± 25% Lower measurement limit:0.0.2 P mg/L

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Determination of total organic carbon (TOC)	MSZ EN 1484:1984 Measurement uncertainty: ± 25% Lower measurement limit:0.5 mg/L
Sample preparation for determination of dissolved and suspended matter and total metal content	MSZ 1484-3:2006
Determination of element content (ICP-MS)	EPA 6020B:2014 Measurement uncertainty: ± 25% Lower measurement limit: Tl: 0.003 µ/l Be, Cd, Co: 0.005 µ/l As, Bi, Mo, Ni, Pb, Sb, V: 0.01 µ/l Cr, Li, Sr: 0.05 µ/l B, Cu, Se, Ti, Zn: 0.2 µ/l Al, Fe: 1 µ/l

3.2. Laboratory scale tests for concept development of the liquid and solid-state fermentation process

Target of the 3R liquid fermentation system is the elimination of the phytotoxic compounds from the food industrial WWs and at the same time production of high concentration biomass of the agriculturally beneficial *Trichoderma harzianum* fungus. During the fermentation process, the organic compounds of the WWs and by-products will be converted into recovered and upgraded, safe microbial biomass of added value. Upon finishing the liquid fermentation, the fungal biomass mycelia will be separated from the liquid phase. The obtained microbial biomass will be used for inoculation of the solid fermentation system where ABC carrier will be added.

The selection of nutrient strategy, concept development and validation of the liquid and solid-state fermentation process were the objectives of the laboratory scale microbiological tests. The selection of optimal industrial fermentation media is critically important for cost efficient production of fungal microbiological products on a large scale. Therefore, economics will play a key role in the laboratory scale fermentation medium selection. Inexpensive and readily available materials will be selected.

The following nutrient selection strategies were applied:

- Cost efficiently viable in the industry.
- Food industrial by-products are acceptable, which are optimal or your specific selected stains, industrially available and cost effective.
- Application of expensive and industrial scale not viable cultivation media is restricted for the optimisation process.
- The optimal industrial scale cultivation media consists of only 1-2 components.

Three different scale-up levels were applied: (1) petri-dich agar plate for screening large quantities of samples with different concentrations of food industrial waste water, (2) 500 mL shake flask culture for the laboratory scale liquid fermentation media design and early process modelling, and (3) validation of the liquid fermentation at 3x3 L scale.

3.2.1. Agar plate laboratory tests

The target of the agar plate tests was to evaluate the growing activity and growing morphology of the *Trichoderma harzianum* strains on different composition of agar medium containing variable concentration of different food industrial waste water (acidic whey, corn steep liquor). The inhibitory effects of the different

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concentrations could be modelled for providing a starting point for designing the optimal liquid fermentation medium for the production of the targeted high fungal mycelia for the next stage of solid fermentation.

Three different *Trichoderma harzianum* strains (STA, ST4B, ST5) originating from soil and rhizosphere of the 3R strains collection were used to select suitable strain capable of growing on untreated acidic whey food industrial waste water as a potential liquid fermentation medium. The *Trichoderma* strains were preserved on liquid paraffin oil overlay - agar slant cultures which are stored at 4°C.

Trichoderma harzianum pure cultures were produced on sterilised tomato pulp agar media (120 g tomato pulp with 28-30ref. %, 8g glucose, 20 g agar per litre of distilled water). The agar plates were inoculated with *Trichoderma harzianum* ST4B, STA and ST5 strains obtained from liquid paraffin oil overlay - agar slant culture. After inoculation the agar plates were stored at 25 °C for seven days.

In the agar plate tests, different agar medium compositions were prepared and steam sterilised at 1 atm, 121 °C, 30 min. The agar plates were inoculated with *Trichoderma harzianum* ST4B strain obtained from seven-day solid agar culture on tomato pulp agar medium. After inoculation, the agar plates were stored at 25 °C. The morphology and sporulation activity of the strains were visually observed. The growth radius of the colonies was measured after an incubation period of two and three days.

3.2.1.1. Agar plate test for comparison of Trichoderma harzianum strain growth on tomato agar and tomato-corn steep liquor agar media

This test was carried out on agar plates for comparison of the colony morphology and growing intensity of the *Trichoderma harzianum* ST4B strain on tomato pulp agar medium (120 g/L tomato pulp with 28-30ref. %, 8 g/L glucose, 20 g/L agar) prepared with 0-10-20-40-60-80-100% v/v corn steep liquor which was diluted with distilled water.

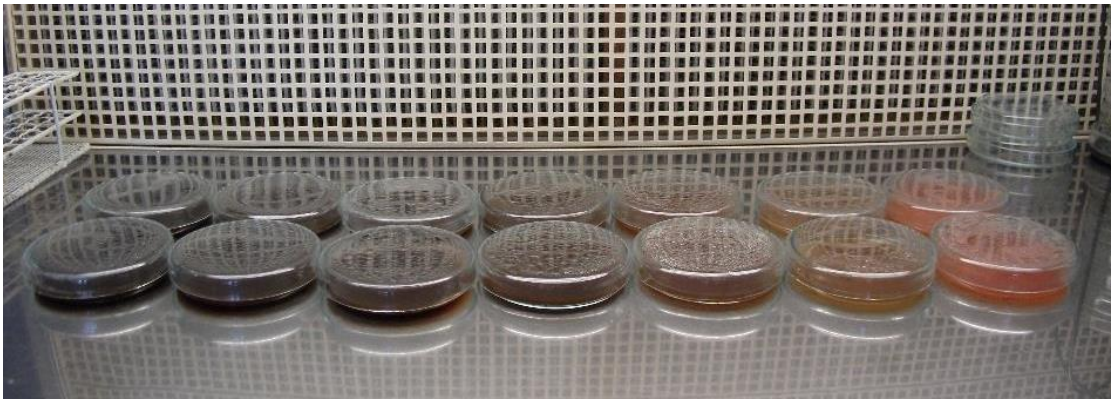

Figure 3-1. Preparation of tomato-pulp agar prepared with different concentrations of corn steep liquor (10-20-40-60-80-100% v/v).

3.2.1.2. Agar plate test for comparison of Trichoderma harzianum strains growth on different growing media

This test was carried out on agar plates for comparison of the colony morphology and growing intensity of the *Trichoderma harzianum* ST4B strain on three different solid growing media: tomato pulp agar (120 g tomato pulp with 28-30ref. %, 8 g glucose, 20 g agar per litre of distilled water), potato agar (32 g potato powder, 8 g glucose, 20 g agar per litre of distilled water), malt agar (24 g malt syrup, 20 g agar per litre of distilled water) and on acidic whey agar (20 g agar per litre of untreated acidic whey).

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3.2.1.3. Agar plate test for comparison of Trichoderma harzianum strains growth on potato-acidic whey media

The first test was carried out on agar plates for comparison of the colony morphology and growing intensity of the three different *Trichoderma harzianum* strains (ST4B, STA, ST5) on potato agar (32 g/L potato powder, 8 g/L glucose, 20 g/L agar) prepared with 0-50-100% v/v untreated acidic whey food industrial waste water which was diluted with distilled water.

The second test was carried out on agar plates for comparison of the colony morphology and growing intensity of the *Trichoderma harzianum* ST4B strain on acidic whey agar (20 g agar per litre untreated acidic whey) supplemented with potato powder at three different concentrations (8-16-24 g/L).

3.2.1.4. Agar plate test for comparison of Trichoderma harzianum strains growth on malt-acidic whey media

This test was carried out on agar plates for comparison of the colony morphology and growing intensity of the three different *Trichoderma harzianum* strains (ST4B, STA, ST5) on malt agar (24 g/L malt syrup, 20 g/L agar) prepared with 0-50-100% V/V untreated acidic whey which was diluted with distilled water.

3.2.1.5. Agar plate test for comparison of Trichoderma harzianum strains growth on tomato pulp-acidic whey media

The first test was carried out on agar plates for comparison of the colony morphology and growing intensity of the three different *Trichoderma harzianum* strains (ST4B, STA, ST5) on tomato pulp agar (120 g/L tomato pulp with 28-30ref. %, 8 g/L glucose, 20 g/L agar) prepared with 0-25-50-75-100 % v/v untreated acidic whey which was diluted with distilled water.

The second test was carried out on agar plates for comparison of the colony morphology and growing intensity of the *Trichoderma harzianum* ST4B strain on tomato pulp agar (120 g/L tomato pulp with 28-30ref. %, 8g/L glucose, 20 g/L agar) prepared with 0-20-40-60-80-100 % v/v untreated acidic whey which was diluted with distilled water.

The third test was carried out on agar plates for comparison of the colony morphology and growing intensity of the *Trichoderma harzianum* ST4B strain on acidic whey agar (20 g agar per litre untreated acidic whey) supplemented with tomato pulp with 28-30 ref % at six different concentrations (20-40-60-80-100-120 g/L).

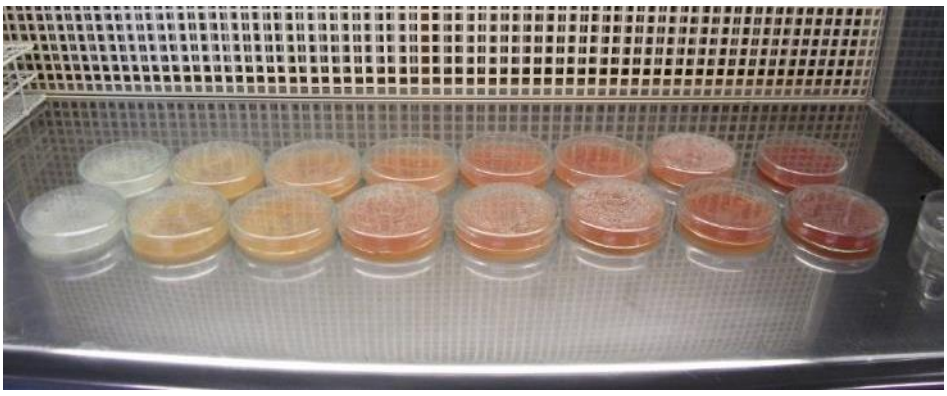

Figure 3-2. Preparation of acidic whey agar plates supplemented with tomato pulp at six different (20-40-60-80-100-120 g/L) concentration.

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3.2.2. Shake flask culture method for fermentation medium design

Shake-flask culture method was set-up for the laboratory scale liquid fermentation media design, early process modelling and bioprocess development of the liquid fermentation.

Bioblock Scientific shaker was used with shaking speed of 45 L/min. Capacity volume of shakers: 500 mL.

Tomato pulp agar medium (120 g tomato pulp with 28-30ref. %, 8 g glucose per litre of distilled water) was used for production of Trichoderma spore suspension for inoculation of shake flasks. The tomato-pulp agar medium was steam sterilised at 1 atm, 121°C, 30 min. The agar plates were inoculated with *Trichoderma harzianum* ST4B strains obtained from liquid paraffin oil overlay - agar slant culture After inoculation the agar plates were stored at 25˚C for seven days. 20 mL of sterilised distilled water was pipetted into the 7-day agar cultures, the spores were suspended into the distilled water.

The first test was carried out in Erlenmeyer flasks with 500 mL capacity for evaluation of the growing intensity of *Trichoderma harzianum* ST4B strain in tomato pulp liquid medium (120 g/L tomato pulp with 28-30ref. %, 8 g/L glucose) prepared with 0-50-75-100 % v/v untreated acidic whey which was diluted with distilled water. The different liquid mediums were steam sterilised at 1 atm, 121 °C, 30 min.

The second test was carried out in Erlenmeyer flasks with 500 mL capacity for evaluation of the growing intensity of *Trichoderma harzianum* ST4B strain in potato liquid medium (32 g/L potato powder, 8 g/L glucose) prepared with 0-50-75-100 % v/v untreated acidic whey which was diluted with distilled water.

Each Erlenmeyer flasks were inoculated with 10 mL of *Trichoderma harzianum* ST4B spore suspension obtained from seven-day solid agar cultures on tomato pulp agar medium. After inoculation the Erlenmeyer flasks were placed on the Bioblock Scientific shaker with shaking speed of 45 L/min at 25°C.

After a seven-day fermentation period, the obtained mycelia were separated from the liquid medium by filtration. The weight of the obtained wet biomass was measured. The determination of water content was based on loss on drying method. In this method, a wet sample was weighed on a balance, dried at 110 °C for 1 hour and loss of weight was measured with a balance. After determining the water content, the mass of the dried biomass was calculated. The mycelia growth was also checked by microscopic observation.

3.2.3. Laboratory scale validation of the liquid fermentation at 3x3 L scale.

The objective of this work is the laboratory scale validation of the liquid fermentation at 3x3 L scale. Tomato pulp agar medium (120 g tomato pulp with 28-30ref. %, 8g glucose per litre of distilled water) was used for production of Trichoderma spore suspension for inoculation of the 3x3 L liquid fermenters. The tomato-pulp agar medium was steam sterilised at 1 atm, 121 °C, 30 min. The agar plates were inoculated with *Trichoderma harzianum* ST4B strains obtained from liquid paraffin oil overlay - agar slant culture. After inoculation the agar plates were stored at 25 °C for seven days. 20 mL of sterilised distilled water was pipetted onto the 7-day agar cultures, the spores were suspended into the distilled water

Tomato-pulp fermentation medium (120 g/L tomato pulp with 28-30ref. %, 8 g/L glucose) was tested which prepared with 50% v/v and 75% v/v untreated acidic whey diluted with distilled water. For each fermentation test 3x3 L of liquid mediums were prepared directly in the fermenters and sterilised together with the ventilation flasks and pipes in autoclave at 1 atm, 121°C for 30 min. After sterilisation, the fermenters were cooled down to 25°C.

Each fermenter was inoculated with 2x20 mL spore suspension of *Trichoderma harianum* ST4B strain obtained from seven-day solid agar culture on tomato-pulp agar medium.

Fermentation condition: Batch fermentation for three days duration the fungal mycelia is sensitive for mechanical agitation therefore the fermentation vessels were air stirred Fermentation temperature: 22°C. For adequate mixing and oxygen supply 0.2-0.5 L/min·L liquid air flow was applied. The samples were microscopically observed and purity check tests on tomato-pulp agar were also performed. The weight of the obtained wet biomass was measured. The determination of water content was based on loss on drying method.

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Figure 3-3. Layout of the Chemoferm lab fermenters with 3x3 L capacity.

3.2.4. Laboratory scale tests for development of solid-state fermentation for BioPhosphate production

The objective of the laboratory scale solid state fermentation is the optimisation of integrated solid-state fermentation and formulation, where vision of industrial scale will be evaluated:

- STAGE 1: liquid phase fermentation for inoculum production, input for stage 2,
- STAGE 2: solid state fermentation for obtaining large amount of high-quality spores and internal colonisation on the internal surface of the ABC carrier for production of the BioPhosphate multifunctional biofertiliser product.
- AfterSolid State Fermentation and Formulation (SSFF) cycle: a slow drying and moisture decrease will be done.

In the laboratory scale solid state fermentation tests the separated biomass from the 3x3 L liquid fermenter was used. The following five different tests were performed:

Test 1:		Test 2:	
Animal bone char	100 g	Animal bone char	100 g
Tomato pulp	50 g	Tomato pulp	50 g
Glucose	10 g	Glucose	10 g
Fungal biomass	50 g	Fungal biomass	50 g
Distilled water	100 mL	Acidic whey	100 mL

Test 3:		Test 4:	
Animal bone char	100 g	Animal bone char	100 g
Tomato pulp	50 g	Tomato pulp	50 g
Glucose	10 g	Glucose	10 g
Fungal biomass	50 g	Fungal biomass	50 g
Distilled water	50 mL	Distilled water	20 mL
Acidic whey	50 mL	Acidic whey	80 mL

Test 5	
Animal bone char	100 g
Tomato pulp	50 g
Glucose	10 g
Fungal biomass	50 g

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Distilled water	80 mL
Acidic whey	20 mL

The fungal growth was visually observed. Colony Forming Units (CFU) test was performed to estimate the number of viable propagules of strain per unit mass. To determine the number of CFU, a sample was prepared and spread or poured uniformly on a surface of an agar plate and then incubated at some suitable temperature for several days. The colonies formed were counted. CFU is not a measure for individual cells or spores as a colony may be formed from a single or a mass of cells or spores.

For determination of CFU Bengal rose agar medium (5g Peptone, 10 g D-Glucose, 1 g KH_2PO_4, 0.5 g $MgSO_4$, 0.05 g Bengal rose, 0.10g Chloramphenicol, 15g agar per litre distilled water, pH: 7.2) was used. Agar plates were incubated at 22 °C for 3-4 days.

3.3. Laboratory scale experiments for adsorption tests of the dairy WW stream acidic whey

The task objective was to study and optimise the operational conditions of the technology proposed in WalLAB for ABC adsorption of dairy food industrial acidic whey WW. Biofarm Agri Research Station is located in the central Transdanubia region Hungary, that region is concentrated cattle farming agricultural and dairy food processing area.

The unique high macroporous Animal Bone Char (ABC) considered to be applied at TRL5 research level, which is an additional support treatment option for the main fermentation process, aiming to find viable upcycling solution for this problematic WW stream. The objective of the adsorption tests was to treat the liquid phase fermentation medium originated from the biotech line and direct treatment for the food industrial WW acidic whey for achieving technological irrigation clean water used for moisture control for the co-composting main application.

Two types of liquid streams processed, such as:

a) Direct adsorption processing of the acidic whey dairy WW stream and
b) Adsorption processing of the separated fermentation liquid residuals.

Figure 3-4 shows the different adsorption materials applied for tests.

a b c

Figure 3-4. Adsorption materials applied for tests. A: ABC mesh 4 sized, b: ABC mesh 8 sized, c: ABC mesh 20 sized

ABC can be thermally regenerated 3-4 times, thereafter, reused as an economic biofertiliser with high phosphorus concentration. The two (a. and b.) liquid treatment options are providing wide range of flexible and economically viable processing options to develop multi-functional biofertilisers and extract irrigation clean water that are upcycled from the dairy WW stream and used for upgrade of co-composted materials at onsite farm level to close the nutrient cycle.

The multifunctional ABC adsorbent used the dairy food industrial WW acidic whey recovery and upcycling solution, consisting of two applications:

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1) Solid microbiological carrier for fungus adaptations during solid state fermentation as main treatment line, which biofertiliser is also containing economically high P nutrient density (35% P_2O_5).
2) Final treatment of the separated liquid effluent from liquid fermentation process. The end-product is a clean irrigation water that will be reused for cattle manure based compost moisturising at regional dairy farmers to close the cycle and possibly horticultural or other industrial uses.

The surface area, a unique macroporous structure, alkaline character and a high degree of surface reactivity make the ABC versatile adsorbent in the case of food industrial WW acidic whey with macromolecular organic pollutants and rather low pH. ABC is consisting of 35% CaO therefore the material has significant pH adjustment effect. Additionally food grade sodium bicarbonate powder is also used (sodium hydrogen carbonate CAS 144-33-8, pH 8.3, water soluble and forming alkaline solution). The pH is successfully adjusted from 4.5 to 7.

Adsorptions tests and experiments made to measure adsorption equilibrium and kinetics from solutions. It consists of the addition of a known mass of sample to a fixed volume of liquid at an initial concentration. The adsorption amount is calculated by subtracting the number of remaining molecules at the adsorption equilibrium from the number of introduced molecules. Figure 3-5 shows the adsorption net applied for tests.

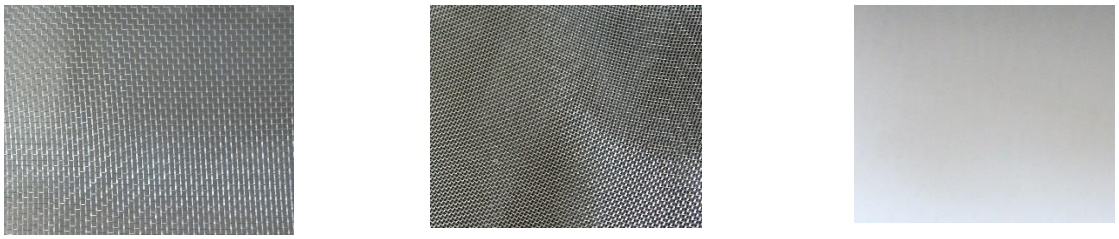

Figure 3-5. Adsorption net applied for the tests, a: filter stainless net mesh 10 sized, b: filter stainless net mesh 20 sized, c: filter micro ceramic mesh 250 sized.

Adsorption tests are useful for generating essential information on the mobility and behaviour of the organic compounds in the food industrial WW acidic whey and can be used to predict or estimate uptake. Adsorption data can also be used for comparative and modelling purposes. Several mechanisms are involved in the process of adsorption, therefore detailed mechanisms cannot be completely defined by this simplified laboratory model. Even if this test cannot cover all the variations and behaviour of the acidic whey substance, it provides practical information on the environmental relevance of the adsorption that is sufficient to design WP3 TRL5.

For pre-treatment mesh 100 (0.149 mm) filter was used. The 2000 cm^3 capacity column filled with 1000 cm^3 (545 g) macroporous ABC adsorber and the adsorption flow rate at 25, 100 or 250 mL/min was tested and finally 100 mL/min was determined. As the ABC is fully macroporous structured (50 nm-63,000 nm) the flow rate does not significantly impact the saturation and therefore median flow rate is used at TRL5 with low contact time which is an important KPI at scale up. No chemicals were used for the adsorption processing. The tests were executed in normal ambient temperature and pressure conditions. The spent ABC adsorber is thermally regenerated and reused. The lifespan of the adsorbent used was preliminary calculated in laboratory conditions, but the true value lifespan can be determined under TRL5 conditions only. The representative sample of the dairy food industrial WW is supplied by one of the local cheese processing factories with medium scale processing capacity.

The weight of ABC samples in the equations is the oven dry weight. No losses were measured during the test. No chemical reagents were used. The spent ABC can be thermally regenerated and reused for 3-4 times. Then the spent material can be reused as biofertiliser at co-composting processing. Known volumes of solutions of the test substance, at known concentrations are added to ABC samples of known dry weight. The mixture is agitated for 1 minute. The ABC suspensions are then separated by filtration and the aqueous phase is analysed. The amount of substance adsorbed on the ABC sample is calculated as the difference between the amount of test substance initially present in solution and the amount remaining at the end of the experiment. The low pH is adjusted and neutralised by the ABC in combination with food grade sodium bicarbonate powder water soluble additive (sodium hydrogen carbonate CAS 144-33-8 at pH 8.3). The high BOD, COD and TOC values are decreased to meet the permissible limits. As of thermal regeneration of the spent adsorber the biofertiliser

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component (that is the main WalNUT concept) is lost. Therefore, the fermentation upcycling process is considered the main treatment line and the adsorption processing supports cycle completion. The reproducibility, detection limits and recovery results are practically applicable at TRL5 large scale prototype design. The standard laboratory equipment used, is listed below:

a) Tubes or vessels to conduct the experiments made of an inert material, which minimises adsorption of the test substance on its surface.
b) Agitation device.
c) Filtration device (stainless steel filter material) filters of mesh size 10, 20 and 250.
d) Analytical instrumentation, suitable for measuring the concentration of the test chemical.
e) Laboratory oven, capable of maintaining a temperature from 103 ° to 110 °C.

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4. Results and discussion

4.1. Results of the analytical characterisation of the acidic whey waste water feedstock

Date of sampling: December 03, 2022

Date of analysis: December 04, 2022 – December 15, 2022

Table 4-1: Result of the analytical characterisation of the untreated acidic whey feedstock. Date of sampling: December 03, 2022

Parameters	Dilution	Measure	Units
CODs	-	65900	mg/L
BOD_5	-	17100	mg/L
TOC	-	21850	mg/L
NH_4^+	-	161	mgN/L
NO_3^-	-	16	mgN/L
NO_2^-		<0.003	mgN/L
Total organic nitrogen		512	mgN/L
Total nitrogen	-	689	mgN/L
PO_4^{3-}	-	1917	mg/L
Total phosphorous	-	814	mgP/L
SO_4^{2-}	-	160	mg/L
TSS	-	1073	mg/L
Alkalinity	-	n/a	
Electrical conductivity (25°C)	-	8350	µS/cm
pH	-	4.49	-
Potentially Toxic Elements (PTEs)			
	Dilution	Measure	Units
Aluminium	-	0.161	mg/L
Arsenic	-	<0.001	mg/L
Boron	-	0.237	mg/L
Beryllium	-	<0.001	mg/L
Bismuth	-	<0.001	mg/L
Cadmium	-	<0.001	mg/L
Cobalt	-	<0.001	mg/L
Chromium	-	<0.001	mg/L
Copper	-	0.021	mg/L
Iron	-	0.185	mg/L
Lanthanum	-	<0.001	mg/L
Lithium	-	0.003	mg/L
Molybdenum	-	0.031	mg/L
Nickel	-	0.002	mg/L
Lead	-	<0.001	mg/L

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Rubidium	-	<0.001	mg/L
Antimony	-	<0.001	mg/L
Selenium	-	0.003	mg/L
Strontium	-	0.511	mg/L
Titanium	-	0.002	mg/L
Thallium	-	<0.001	mg/L
Vanadium	-	<0.001	mg/L
Zinc	-	2.960	mg/L
Organic micropollutants (OMP)			
	Dilution	Measure	Units
Ciprofloxacin	-	not relevant	Remarks: Pharmaceuticals are not detectable in the dairy industrial acidic whey.
Sulfadiazine			
Oxytetracycline			
Sulfamethoxazole			
Azithromycin			
Trimethoprim			
Citalopram			
Fluoxetine			
Diazepam			
Carbamazepine			
Methiocarb			

Table 4-2: Result of the analytical characterisation of acidic whey sample. Date of sampling: January 17, 2023

Parameters	Dilution	Measure	Units
CODs	-	66800	mg/L
BOD_5	-	17500	mg/L
TOC	-	22400	mg/L
NH_4^+	-	162	mgN/L
NO_3^-	-	16	mgN/L
NO_2^-		<0.003	mgN/L
Total organic nitrogen		508	mgN/L
Total nitrogen	-	686	mgN/L
PO_4^{3-}	-	1930	mg/L
Total phosphorous	-	837	mgP/L
SO_4^{2-}	-	161	mg/L
TSS	-	1080	mg/L
Alkalinity	-	n/a	
Electrical conductivity (25°C)	-	8360	µS/cm
pH	-	4.54	-
PTEs			
	Dilution	Measure	Units
Aluminium	-	0.167	mg/L
Arsenic	-	<0.001	mg/L

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Boron	-	0.240	mg/L
Beryllium	-	<0.001	mg/L
Bismuth	-	<0.001	mg/L
Cadmium	-	<0.001	mg/L
Cobalt	-	<0.001	mg/L
Chromium	-	0.002	mg/L
Copper	-	0,020	mg/L
Iron	-	0.173	mg/L
Lanthanum	-	<0.001	mg/L
Lithium	-	0.006	mg/L
Molybdenum	-	0.038	mg/L
Nickel	-	0.003	mg/L
Lead	-	0.002	mg/L
Rubidium	-	<0.001	mg/L
Antimony	-	<0.001	mg/L
Selenium	-	0.002	mg/L
Strontium	-	0.626	mg/L
Titanium	-	0.022	mg/L
Thallium	-	<0.001	mg/L
Vanadium	-	<0.001	mg/L
Zinc	-	2.930	mg/L
Organic micropollutants (OMP)			
	Dilution	Measure	Units
Ciprofloxacin	-	not relevant	Remarks: Pharmaceuticals are not detectable in the dairy industrial acidic whey.
Sulfadiazine			
Oxytetracycline			
Sulfamethoxazole			
Azithromycin			
Trimethoprim			
Citalopram			
Fluoxetine			
Diazepam			
Carbamazepine			
Methiocarb			

Observations: Slightly greenish-yellowish colour translucent liquid.

Pharmaceuticals are not detectable in the dairy industrial acidic whey. 3R use acidic whey WW, which have been lawfully produced from continuously controlled / inspected raw milk and regularly tested by the accredited Dairy Research Institute, furthermore Food Research and Testing and Raw Milk Classification Laboratory. The accreditation is according to EN ISO / IEC 17025. The raw milk that is lawfully used for industrial processing is free from inhibitors: pharmaceuticals and PTEs. Any raw milk that contains not allowable levels of pharmaceuticals and PTEs must be discarded by law, may not enter the food chain and may not be lawfully used for any dairy food industrial processing.

Based on the results of analytical measurement the following lawful tracking parameters of the dairy industrial acidic whey has been selected for 3R WP3 pilot follow up:

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- pH
- COD – dichromate oxygen consumption
- BOD 5days
- TSS
- TOC
- Total nitrogen
- Total phosphorus
- PO_4^{3-}

4.2. Results of the laboratory scale tests for concept development of the liquid and solid-state fermentation process

4.2.1. Results of the small-scale agar plate tests

4.2.1.1. Results of the agar plate test for comparison of Trichoderma harzianum strain growth on tomato agar and corn steep liquor agar media

Table 4-3 and Table 4-4 show the measured colony growth radius of *Trichoderma harzianum* ST4B on tomato pulp agar and corn steep liquor agar at different concentration (0-10-20-40-60-80-100% v/v). The results clearly indicated that corn steep liquor had an inhibitory effect on the fungus. In the case of a concentration of 20% a strong inhibition effect was observed compared to the tomato pulp control agar media. No growth activity was observed at concentrations above 40 % v/v.

Table 4-3: Results of *Trichoderma harzianum* ST4B cultivation of tomato pulp agar medium and corn steep liquor (CSL) agar at different (0-10-20-40-60-80-100% v/v) concentration after 2 days incubation at 25 ^{0}C.

	Tomato pulp agar colony growth radius (cm)		**Corn steep liquor (10% v/v) agar** colony growth radius (cm)		**Corn steep liquor (20% v/v) agar** colony growth radius (cm)		**Corn steep liquor (40% v/v) agar** colony growth radius (cm)	
ST4B	2.5	3.2	1.7	1.8	1.0	1.0	0	0
ST4B	3.8	2.7	1.7	1.6	1.0	1.0	0	0
ST4B average	3.1		1.7		1.0		0	
	Corn steep liquor (60% v/v) agar colony growth radius (cm)		**Corn steep liquor (80% v/v) agar** colony growth radius (cm)		**Corn steep liquor (100% v/v) agar** colony growth radius (cm)			
ST4B	0	0	0	0	0	0		
ST4B	0	0	0	0	0	0		
ST4B average	0		0		0			

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Table 4-4: Results of *Trichoderma harzianum* ST4B cultivation of tomato pulp agar medium and corn steep liquor (CSL) agar at different (0-10-20-40-60-80-100% v/v) concentration after 3 days incubation at 25 ^{0}C.

	Tomato pulp agar colony growth radius (cm)		**Corn steep liquor (10% v/v) agar** colony growth radius (cm)		**Corn steep liquor (20% v/v) agar** colony growth radius (cm)		**Corn steep liquor (40% v/v) agar** colony growth radius (cm)	
ST4B	5.9	4.7	3.2	3.0	1.7	2.0	0	0
ST4B	5.7	4.2	3.2	2.7	2.2	2.0	0	0
ST4B average	5.1		3.0		1.9		0	
	Corn steep liquor (60% v/v) agar colony growth radius (cm)		**Corn steep liquor (80% v/v) agar** colony growth radius (cm)		**Corn steep liquor (100% v/v) agar** colony growth radius (cm)			
ST4B	0	0	0	0	0	0		
ST4B	0	0	0	0	0	0		
ST4B average	0		0		0			

4.2.1.2. Results of the agar plate tests for comparison of Trichoderma harzianum strains growth on different growing media

The results showed that the colony morphologies of *T. harzianum* strains were highly influenced by the composition of the growing media. The results of the nutrient agar tests indicated that *Trichoderma harzianum* strains have intensive white colour hyphal development and sporulation activity (green colour) both on the potato (Figure 4-1) and tomato pulp (Figure 4-2) agar mediums.

Tomato pulp provided sufficient nutrients for all the investigated *Trichoderma harzianum* strains (ST4B, ST5, STA) with an optimal pH 4.5 for *Trichoderma harzianum* strains having a high effective buffer capacity. The mycelial development and the sporulation activity of the investigated Trichoderma fungus were very intensive on tomato pulp therefore high yield of biomass could be achieved. Tomato pulp is a viable nutrient for scale up of the industrial production of fungal mycelia. Therefore, tomato pulp media was used as a control media for the evaluation of the food industrial waste waters.

The hyphal growth on malt agar (Figure 4-3) was much slower than observed on tomato agar and potato agar growing mediums. This means that malt agar is not a good nutrient strategy for liquid fermentation where our target is intensive mycelial growth and high biomass production.

a b

Figure 4-1. *Trichoderma harzianum* ST4B (a) and ST5 (b) strains morphology on potato agar after 8 days incubation time at 25 ^{0}C.

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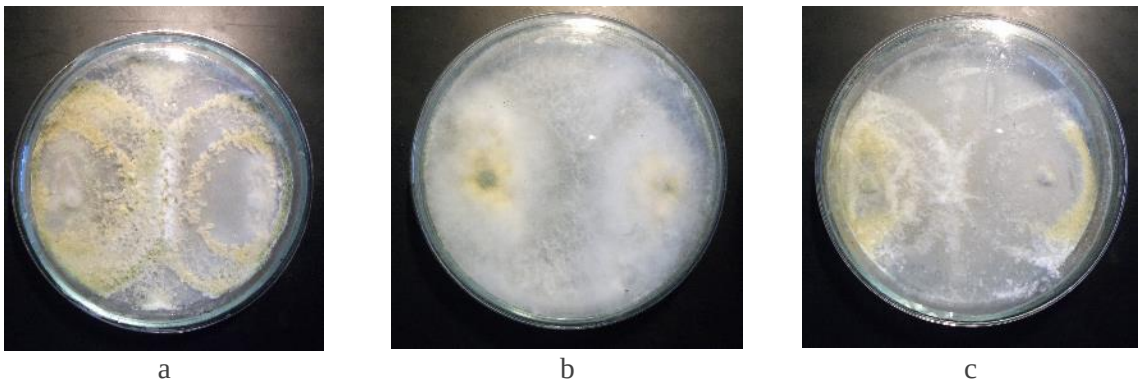
a b c

Figure 4-2. *Trichoderma harzianum* ST4B (a), ST5 (b) and STA (c) strains morphology on tomato agar after 8 days incubation time at 25 ^{0}C.

a b c

Figure 4-3. *Trichoderma harzianum* ST4B (a) ST5 (b) and STA (c) strains morphology on malt agar after 8 days incubation time at 25 ^{0}C.



a b c

Figure 4-4. *Trichoderma harzianum* ST4B (a), ST5 (b) and STA (c) strains morphology on acidic whey agar after 8 days incubation time at 25 ^{0}C.

Figure 4-4 demonstrates that all three tested strains of the *Trichoderma harzianum* (ST4B, ST5, STA) were able to grow on an acidic whey substrate without the addition of other nutrients. They can utilise lactose as a carbon source thanks to lactase enzyme activity of the Trichoderma fungus. On the acidic whey agar yellow pigment was produced which considered secondary metabolites.

The growth radius of the *Trichoderma harzianum* strains on acidic whey agar, tomato pulp agar, malt agar and potato agar were measured after two- and three-days incubation periods and presented in the Table 4-5 and Table 4-6. In the case of acidic whey a slower growth rate and longer sporulation time of the three *Trichoderma harzianum* strains were observed compared to the tomato-pulp agar, potato agar and malt agar growing

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mediums (Table 4-6). A slower growth rate on agar mediums predicts a longer liquid fermentation time for reaching an adequate biomass production. **Therefore, for developing the efficient fermentation nutrient strategy, we need to combine acidic whey with other nutrient strategies.**

Table 4-5: The measured colony growth radius of different *Trichoderma* strains (ST5, ST4B and STA) on tomato-pulp, malt, potato and acidic whey (AW) agar medium after 2 days incubation at 25 °C.

	AW agar colony growth radius (cm)		Tomato pulp agar colony growth radius (cm)		Malt agar colony growth radius (cm)		Potato agar colony growth radius (cm)	
ST5	2.0	2.2	4.0	3.0	2.5	2.3	3.0	3.5
ST5	1.8	2.5	4.5	3.6	-	-	4.0	3.0
ST5 average	**2.1**		**3.8**		**2.4**		**3.4**	
ST4B	3.0	2.5	5.0	3.5	3.7	3.5	4.0	4.0
ST4B	2.0	2.0	3.7	2.8	-	-	3.7	3.5
ST4B average	**2.4**		**3.7**		**3.6**		**3.8**	
STA	2.5	2.0	3.8	3.8	2.8	2.6		
STA	2.3	2.7	3,7	4.0	-	-		
STA average	**2.4**		**3.8**		**2.7**			

Table 4-6: The measured colony growth radius of different *Trichoderma* strains (ST5, ST4B and STA) on tomato-pulp, malt, potato and acidic whey (AW) agar medium after 3 days incubation at 25 ^{0}C.

	AW agar colony growth radius (cm)		Tomato pulp agar colony growth radius (cm)		Malt agar colony growth radius (cm)		Potato agar colony growth radius (cm)	
ST5	2.5	3.2	6.5	4.8	4.5	4.0	4.8	4.9
ST5	3.0	2.9	5.8	5.6	-	-	5.5	5.0
ST5 average	**2.9**		**5.7**		**4.3**		**5.1**	
ST4B	4.5	3.4	6.3	6.8	6.2	6.4	5.5	6.0
ST4B	4.5	3.7	5.5	6.2	-	-	6.2	6.0
ST4B average	**4.0**		**6.2**		**6.3**		5.9	
STA	4.0	4.0	5.8	5.6	5.2	5.2		
STA	4.2	4.0	5.7	5.7	-	-		
STA average	**4.1**		**5.7**		**5.2**			

4.2.1.3. Results of the agar plate tests for comparison of Trichoderma harzianum strains growth on potato-acidic whey media

In this agar plate test potato and acidic whey were combined where acidic whey in different concentration (50-100% v/v) were added to the potato agar medium. Table 4-7**,** Table 4-8 and Figure 4-5 show the results of the measurement of colony growth radius of different *Trichoderma* strains (ST5 and ST4B) on acidic whey, potato and potato-acidic whey agar medium after two- and three-days incubation at 25 ^{0}C. **The growth radius of the fungal colonies decreased as the concentration of acid whey increased compared to the potato agar control medium after two and three days of incubation periods. This result indicates that acidic whey has an inhibitory effect on fungal growth. No significant difference was observed when comparing acid whey agar with potato agar containing 100% v/v acid whey.**

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Table 4-7: The measured colony growth radius of different *Trichoderma* strains (ST5 and ST4B) on acidic whey, potato and potato-acidic whey agar medium after 2 days incubation at 25 °C.

	AW agar colony growth radius (cm)		Potato agar -AW 0% v/v colony growth radius (cm)		Potato-AW 50% v/v agar colony growth radius (cm)		Potato-AW 100% v/v agar colony growth radius (cm)	
ST5	2.0	2.2	3.0	3.5	2.5	3.0	1.8	1.5
ST5	1.8	2.5	4.0	3.0	2.3	2.5	1.7	1.5
ST5 average	**2.1**		**3.4**		**2.6**		**1.6**	
ST4B	3.0	2.5	4.0	4.0	4.0	4.2	3.5	4.0
ST4B	2.0	2.0	3.7	3.5	4.2	3.8	3.0	2.8
ST4B average	**2.4**		**3.8**		**3.3**		**3.3**	

Table 4-8: The measured colony growth radius of different *Trichoderma* strains (ST5 and ST4B) on acidic whey, potato and potato-acidic whey (0-50-100 %v/v) agar medium after 3 days incubation at 25 ^{0}C.

	AW agar colony growth radius (cm)		Potato agar 0% v/v agar colony growth radius (cm)		Potato-AW 50% v/v agar colony growth radius (cm)		Potato-AW 100% v/v agar colony growth radius (cm)	
ST5	2.5	3.2	4.8	4.9	3.2	3.4	2.7	2.8
ST5	3.0	2.9	5.5	5.0	3.8	4.2	2.9	2.5
ST5 average	**2.9**		**5.1**		**3.7**		**2.7**	
ST4B	4.5	3.4	6.3	6.2	5.5	6.0	5.2	4.5
ST4B	4.5	3.7	6.2	6.0	6.2	6.0	4.0	4.2
ST4B average	**4.0**		**6.2**		**5.9**		**4.4**	

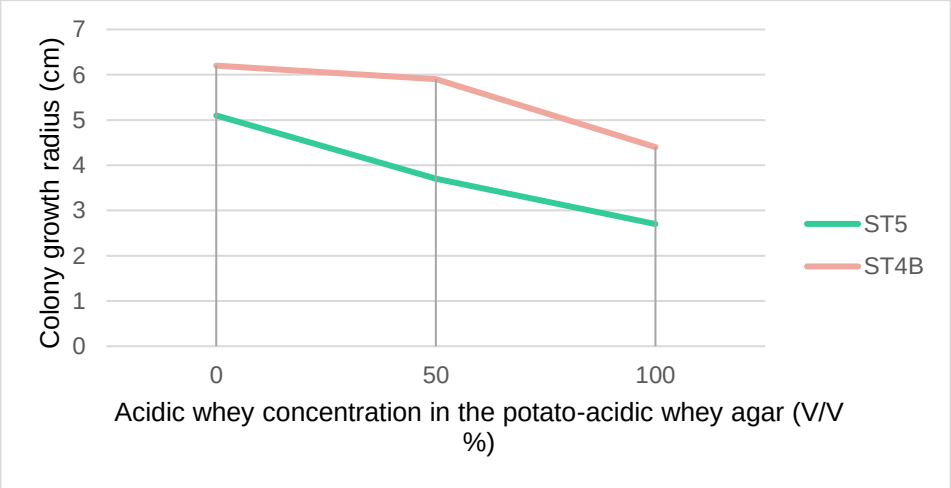

Figure 4-5. Changes in the colony growth radius of *Trichoderma harzianum* ST4B-ST5 strains on potato-acidic whey (AW) agar medium contains acidic whey in 0-50-100% v/v concentration three days incubation period at 25 °C.

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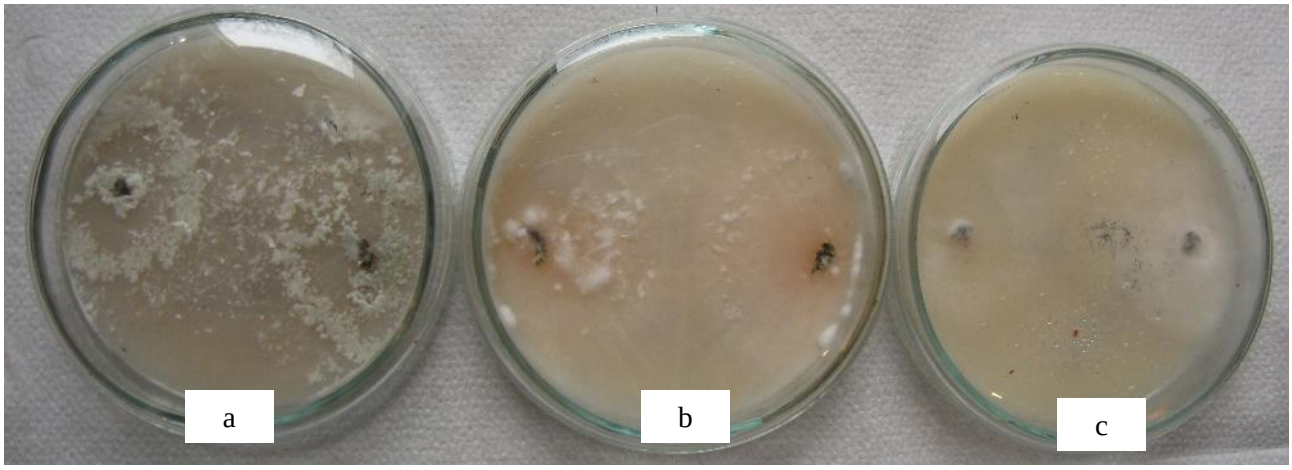

Figure 4-6. Results of *Trichoderma harzianum* ST4B cultivation on potato agar medium containing different concentration of untreated acidic whey after 4 days incubation at 25 ^{0}C. a: 0% v/v acidic whey, b: 50% v/v acidic whey, c: 100 % v/v acidic whey.

Figure 4-6 shows the morphological view of the *Trichoderma harzianum* ST4B cultivation on potato agar medium containing different concentrations of untreated acidic whey after 4 days of incubation at 25 °C. In the case of potato agar with 0 % v/v acid whey, sporulation started after 4 days of incubation, on the contrary, the fungus was still in the phase of mycelial growth, when the potato agar contains 50 % v/v and 100% v/v acid whey. In the second agar plate test, potato powder was added in three different concentrations (8, 16 and 24 g/L) to the 100 % v/v acidic whey medium. The results of the measurement of the growth radius of fungal colonies are presented in the Table 4-9 and Table 4-10. The growth radius of the fungal colonies was slightly increased by the increasing concentration of the potato power, but it was still significantly lower compared to the control. **According to the results of the potato-acidic whey agar plate tests, the concentrated acidic whey waste water must be diluted in order to obtain a suitable liquid fermentation medium.**

Table 4-9: The measured colony growth radius of *Trichoderma* strains ST4B strain on potato- and potato-acidic whey agar medium contains 8-16-24 g/L potato after 2 days incubation at 25 °C.

	Potato agar (32 g/L potato powder)colony growth radius (cm)		AW agar + 8 g/L potato powder colony growth radius (cm)		AW agar + 16 g/L potato powder colony growth radius (cm)		AW agar + 24 g/L potato powder colony growth radius (cm)	
ST4B	3.5	3.8	0.7	1.0	1.0	1.2	1.5	1.7
ST4B	2.2	3.2	0.7	0.8	1.2	1.2	1.7	2.0
ST4B average	3.2		1.1		1.2		1.7	

Table 4-10: The measured colony growth radius of *Trichoderma* strains ST4B strain on potato- and potato-acidic whey agar medium contains 8-16-24 g/L potato after 3 days incubation at 25 °C.

	Potato agar (32 g/L potato powder) colony growth radius (cm)		AW agar + 8 g/L potato powder colony growth radius (cm)		AW agar + 16 g/L potato powder colony growth radius (cm)		AW agar + 24 g/L potato powder colony growth radius (cm)	
ST4B	5.6	6.0	2.7	3.2	2.8	3.2	3.5	3.7
ST4B	5.6	6.0	2.6	2.8	3.2	3.2	3.7	3.8
ST4B average	5.8		2.8		3.1		3.7	

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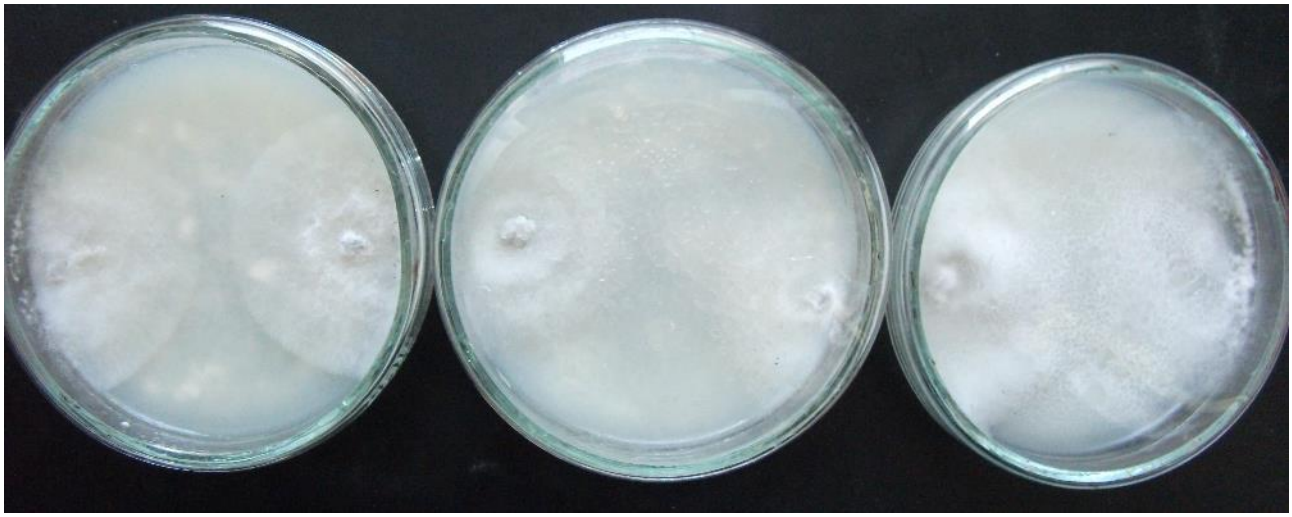

Figure 4-7: Results of *Trichoderma harzianum* ST4B cultivation on potato- and potato-acidic whey agar medium contains 8-16-24 g/L potato after 5 days incubation at 25 °C.

Figure 4-7 shows the results of the cultivation of *Trichoderma harzianum* ST4B cultivation on potato- and potato-acidic whey agar medium containing 8-16-24 g/L potato after 5 days incubation at 25 °C. A higher growth intensity can be observed at a higher potato powder concentration. The white colour indicates that the fungus is still in the mycelial development stage, and the sporulation has still not started.

4.2.1.4. Results of the agar plate tests for comparison of Trichoderma harzianum strains growth on malt-acidic whey media

In this agar plate test malt syrup and acidic whey were combined and acidic whey in different concentration (50-100% v/v) was added to the malt syrup agar medium. Table 4-11 and Table 4-12 show the colony growth radius of different *Trichoderma* strains (ST5, STA and ST4B) on malt- and malt-acidic whey agar medium contains 50 and 100% v/v acidic whey after incubations of two and three days at 25 °C. According to the results **when 100 % v/v acid whey was added to the malt agar, smaller growth colony radius was measured compared to 50% v/v acid whey concentration**. We did not find any significant difference in the colony growth radius when malt agar was compared to malt and acidic whey agar at a concentration of 50% v/v. So, it can be suggested that **malt agar is not a good nutrient strategy when intensive mycelial growth and high biomass production are our goals.**

Table 4-11: The measured colony growth radius of *Trichoderma* ST5, ST4B and STA strains on malt- and malt-acidic whey agar medium contains 50 and 100% v/v acidic whey after 2 days incubation at 25 °C.

	Malt agar (0% v/v AW) colony growth radius (cm)		Malt-AW (50% v/v) agar colony growth radius (cm)		Malt-AW (100% v/v) agar colony growth radius (cm)	
ST5	2.5	2.3	2.5	2.0	1.8	1.7
ST5	-	-				
ST5 average	**2.4**		**2.3**		**1.8**	
ST4B	3.7	3.5	3.2	3.6	3.0	3.0
ST4B	-	-	-	-	-	-
ST4B average	**3.6**		**3.4**		**3.0**	
STA	2.8	2.6	2.7	3.2	2.5	2.6
STA	-	-	-	-	-	-
STA average	**2.7**		**3.0**		**2.6**	

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Table 4-12: The measured colony growth radius of *Trichoderma* ST5, ST4B and STA strains on malt- and malt-acidic whey agar medium contains 50 and 100% v/v acidic whey after 3 days incubation at 25 °C.

	Malt agar (0% v/v AW) colony growth radius (cm)		Malt-AW (50% v/v) agar colony growth radius (cm)		Malt-AW (100% v/v) agar colony growth radius (cm)	
ST5	4.5	4.0	4.5	4.0	3.0	3.3
ST5	-	-	-	-	-	-
ST5 average	**4.3**		**4.3**		**3.2**	
STB	6.2	6.4	6.0	6.0	5.2	4.6
ST4B	-	-	-	-	-	-
ST4B average	**6.3**		**6.0**		**4.9**	
STA	5.2	5.2	5.5	5.8	4.0	4.2
STA	-	-	-	-	-	-
STA average	**5.2**		**5.7**		**4.1**	

4.2.1.5. Results of the agar plate tests for comparison of Trichoderma harzianum strains growth on tomato pulp-acidic whey media

In this agar plate test tomato and acidic whey were combined where acidic whey in different concentration (0-25-50-75-100% v/v) were added to the tomato-pulp agar medium and inoculated with *Trichoderma harzianum* ST4B, ST5and STA strains.

Table 4-13, Table 4-14 and Figure 4-8 show the results of the measurement of the growth radius of *Trichoderma harzianum* ST4B, ST5, and STA strains cultivated on tomato pulp agar medium containing different concentrations (0-25-50-75-100% v/v) of untreated acidic whey (AW) after incubation of two and three days. **The growth radius of the** *Trichoderma harzianum* (ST4B, STA, ST5) **fungal colonies decreased in all cases, with the increase of the concentration of acidic whey** compared to the tomato pulp agar control medium after two and three days of incubation. **This result indicates that acidic whey has an inhibitory effect on fungal growth. The highest growing activity was observed for the ST4B followed by STA and ST5 (**Figure 4-8).

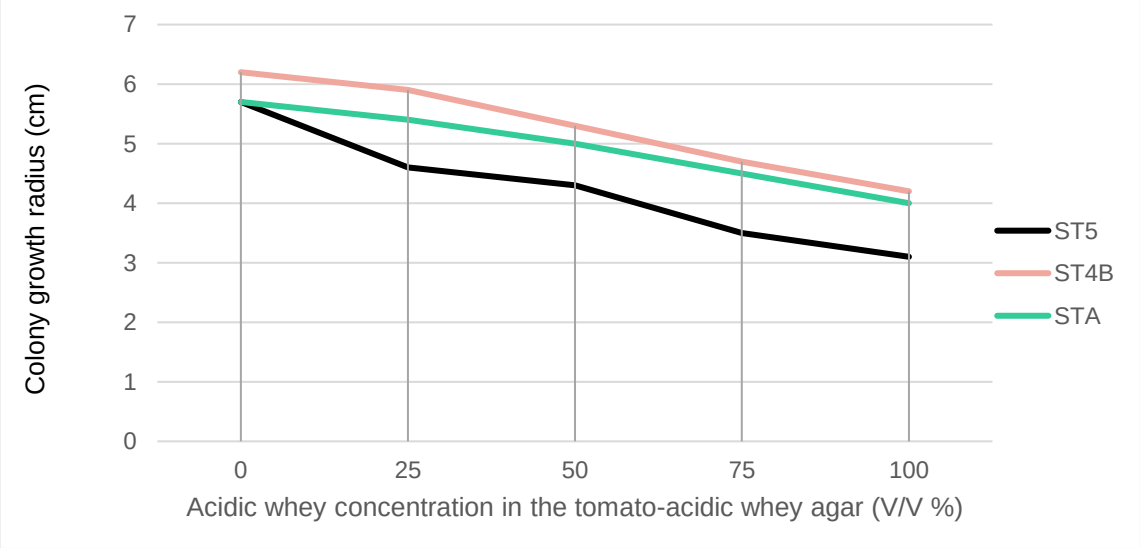

Figure 4-8. Changes in the colony growth radius of *Trichoderma harzianum* ST4B-STA-ST5 strains on tomato pulp-acidic whey (AW) agar medium contains acidic whey in 0-20-40-60-80-100% v/v concentrations after three days incubation period at 25 °C.

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Table 4-13: Results of *Trichoderma harzianum* ST4B, ST5and STA cultivation of tomato pulp agar medium containing different concentration (0-25-50-75-100% v/v) of untreated acidic whey (AW) after 2 days incubation at 25 °C.

	AW agar colony growth radius (cm)		**Tomato-AW (0 % v/v) agar** colony growth radius (cm)		**Tomato-AW (25% v/v) agar** colony growth radius (cm)		**Tomato-AW (50% v/v) agar** colony growth radius (cm)		**Tomato-AW (75% v/v) agar** colony growth radius (cm)		**Tomato-AW (100% v/v) agar** colony growth radius (cm)	
ST5	2.0	2.2	4.0	3.0	2.7	3.0	2.6	2.3	2.0	2.0	1.9	1.6
ST5	1.8	2.5	4.5	3.6	2.0	3.2	2.4	2.5	2.0	1.8	1.8	2.0
ST5 average	2.1		3.8		2.7		2.5		2.0		1.8	
ST4B	3.0	2.5	5.0	3.5	3.7	3.8	3.6	3.2	2.5	2.5	2.6	2.3
ST4B	2.0	2.0	3.7	2.8	3.5	3.8	3.1	3.4	2.5	3.8	2.1	2.4
ST4B average	2.4		3.7		3.7		3.3		2.6		2.4	
STA	2.5	2.0	3.8	3.8	3.0	3.2	2.8	2.7	2.8	2.4	2.1	2.3
STA	2.3	2.7	3.7	4.0	3.5	3.0	2.7	2.9	2.6	2.5	2.0	2.2
STA average	2.4		3.8		3.2		2.8		2.6		2.2	

Table 4-14: Results of *Trichoderma harzianum* ST4B, ST5and STA cultivation of tomato pulp agar medium containing different concentration (0-25-50-75-100 v/v %) of untreated acidic whey (AW) after 3 days incubation at 25 °C.

	AW agar colony growth radius (cm)		**Tomato-AW (0% v/v) agar** colony growth radius (cm)		**Tomato-AW (25% v/v) agar** colony growth radius (cm)		**Tomato-AW (50% v/v) agar** colony growth radius (cm)		**Tomato-AW (75% v/v) agar** colony growth radius (cm)		**Tomato-AW (100% v/v) agar** colony growth radius (cm)	
ST5	2.5	3.2	6.5	4.8	4.2	5.0	4.4	4.2	4.0	3.5	3.0	2.9
ST5	3.0	2.9	5.8	5.6	4.0	5.2	4.1	4.3	3.5	3.0	3.3	3.3
ST5 average	2.9		5.7		4.6		4.3		3.5		3.1	
ST4B	4.5	3.4	6.3	6.8	5.8	5.8	5.4	5.5	4.7	4.8	4.1	4.2
ST4B	4.5	3.7	5.5	6.2	6.0	6.3	5.1	5.2	3.8	5.6	4.4	3.9
ST4B average	4.0		6.2		5.9		5.3		4.7		4.2	
STA	4.0	4.0	5.8	5.6	5.6	5.4	5.1	5.2	4.5	4.6	4.3	3.9
STA	4.2	4.0	5.7	5.7	5.6	5.6	4.9	4.8	4.6	4.2	3.8	4.1
STA average	4.1		5.7		5.4		5.0		4.5		4.0	

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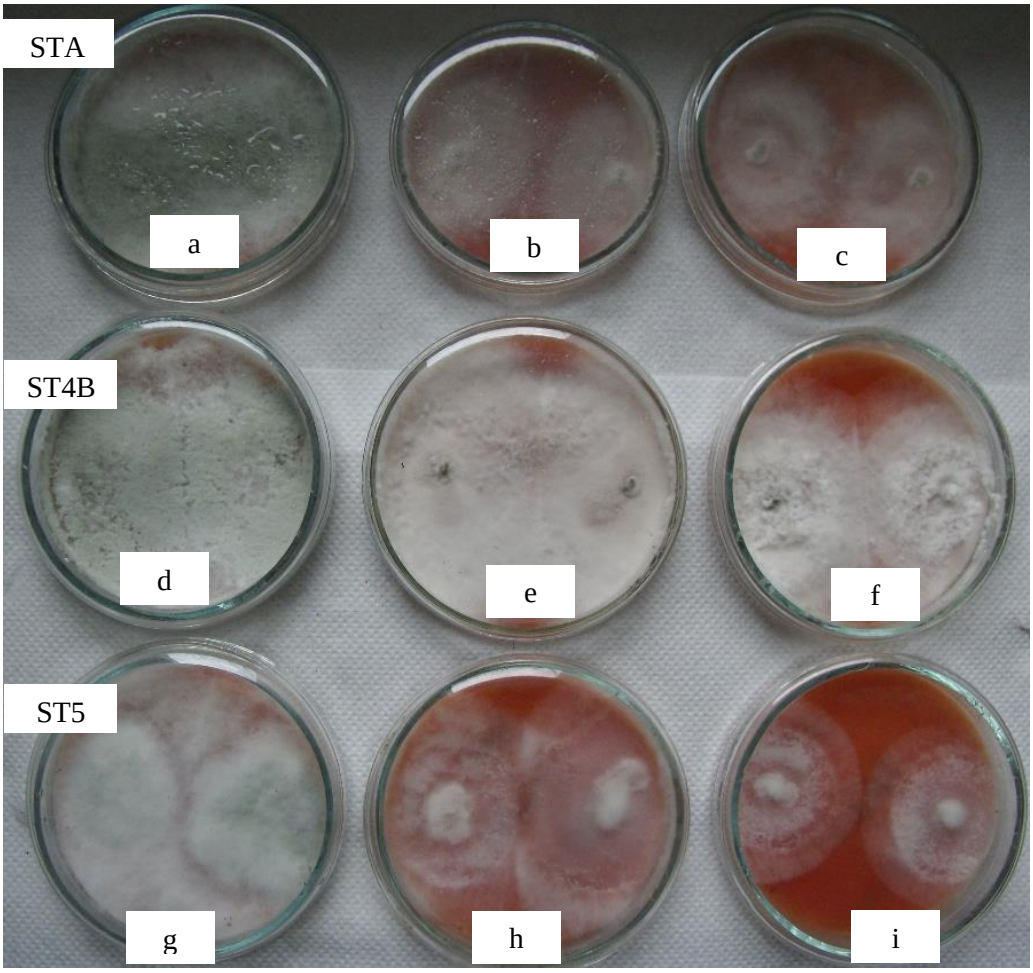

Figure 4-9. Results of *Trichoderma harzianum* STA, ST4B and ST5 cultivation on tomato pulp agar medium containing different concentration of untreated acidic whey after 5 days incubation at 25 °C. a, d, g: 0% v/v acidic whey; b, e, h: 50% v/v acidic whey; c, f, i: 100% v/v acidic whey.

Figure 4-9 shows the results of the cultivation of *Trichoderma harzianum* STA, ST4B and ST5 on tomato pulp agar containing untreated acid whey at concentrations of 0, 50 and 100% v/v after an incubation period of five days. The morphological differences of the fungus on agar plates containing different concentrations of acidic whey are shown in the picture. **In the case of an acid whey concentration of 0 % v/v, the greenish colour indicates that spore formation has already started on the surface of the agar. On the contrary, when 50 and 100% v/v acidic whey was added, the fungal strains were only in the mycelial development phase after 5 days incubation period.**

In the second agar plate test tomato and acidic whey were combined. Acidic whey in different concentrations (0-20-40-60-80-100 % v/v) was added to the tomato-pulp agar medium and inoculated with ST4B strain. Table 4-15, Table 4-16 and Figure 4-10 show the results of the measurement of the growth radius of *Trichoderma harzianum* ST4B strain cultivated on tomato pulp agar medium containing different concentrations (0-20-40-60-80-100% v/v) of untreated acidic whey after incubations of two and three days. **The growth radius of the fungal colonies of the *Trichoderma harzianum* ST4B strain decreased with the increase of the concentration of acidic whey** compared to the tomato pulp agar control medium after both two and three days of incubation. **This result also confirm that acidic whey has an inhibitory effect on fungal growth.** Figure 4-11 shows the morphological development of *Trichoderma harzianum* ST4B cultivated on tomato pulp agar medium containing different concentration (0, 20, 40, 60, 80, 100% v/v) of untreated acidic whey after an incubation period of 4 days. Figure 4-12 shows the results after an incubation period of seven days. The pictures show that as the concentration of acidic whey increases, the growth of the fungus decreased.

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Table 4-15: Results of *Trichoderma harzianum* ST4B cultivation on tomato pulp agar medium containing different concentrations (100-80-60-40-20-0% v/v) of untreated acidic whey (AW) after 2 days incubation at 25 °C.

	Tomato-AW (100 %V/V) agar colony growth radius (cm)		Tomato-AW (80 % V/V) agar colony growth radius (cm)		Tomato-AW (60% V/V) agar colony growth radius (cm)		Tomato-AW (40% V/V) agar colony growth radius (cm)		Tomato-AW (20% V/V) agar colony growth radius (cm)		Tomato-AW (0% V/V) agar colony growth radius (cm)	
ST4B	0.7	0.5	1.2	1.2	2.0	1.6	2.2	2.0	2.7	1.8	3.0	2.4
ST4B	0.9	0.5	0.9	0.8	1.5	1.9	2.2	2.2	2.6	2.5	2.8	2.9
ST4B average	0.7		1.0		1.8		2.2		2.4		2.8	

Table 4-16: Results of *Trichoderma harzianum* ST4B cultivation on tomato pulp agar medium containing different concentration (100-80-60-40-20-0 %v/v) of untreated acidic whey (AW) after 3 days incubation at 25 °C.

	Tomato-AW (100 %V/V) agar colony growth radius (cm)		Tomato-AW (80 % V/V) agar colony growth radius (cm)		Tomato-AW (60% V/V) agar colony growth radius (cm)		Tomato-AW (40% V/V) agar colony growth radius (cm)		Tomato-AW (20% V/V) agar colony growth radius (cm)		Tomato-AW (0% V/V) agar colony growth radius (cm)	
ST4B	2.2	2.6	3.3	3.2	4.5	3.6	5.0	4.8	5.5	5.2	6.0	6.2
ST4B	2.2	1.4	3.2	2.6	4.0	4.6	5.1	4.9	5.2	5.6	6.0	5.2
ST4B average	2.1		3.1		4.2		5.0		5.4		5.9	

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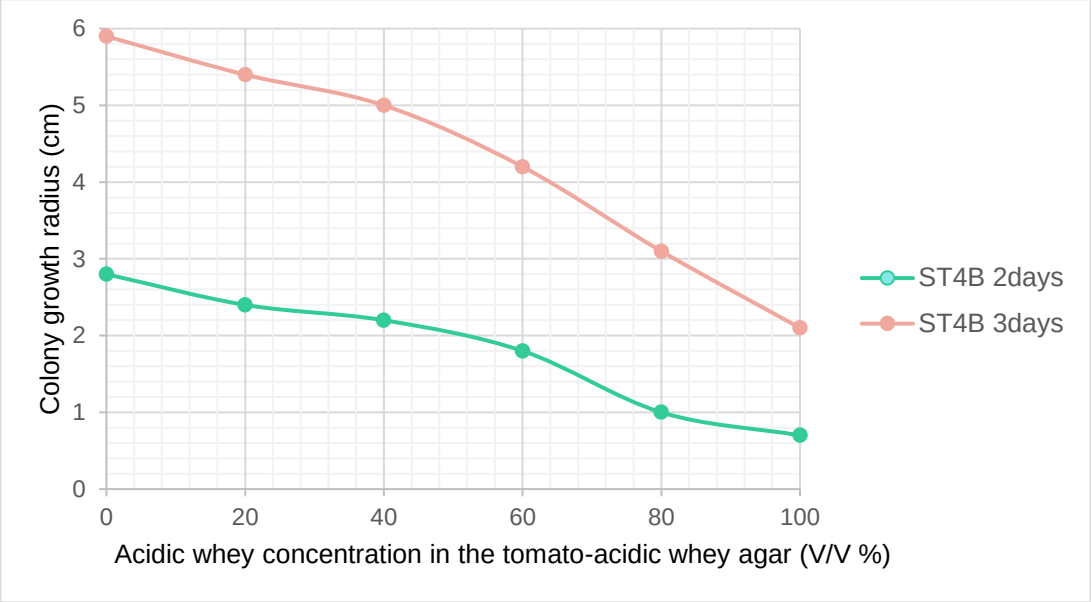

Figure 4-10. Changes in the colony growth radius of *Trichoderma harzianum* ST4B strain on tomato pulp-acidic whey (AW) agar medium contains acidic whey in 0-20-40-60-80-100 % v/v concentration after two- and three-days incubation period at 25 ^{0}C.

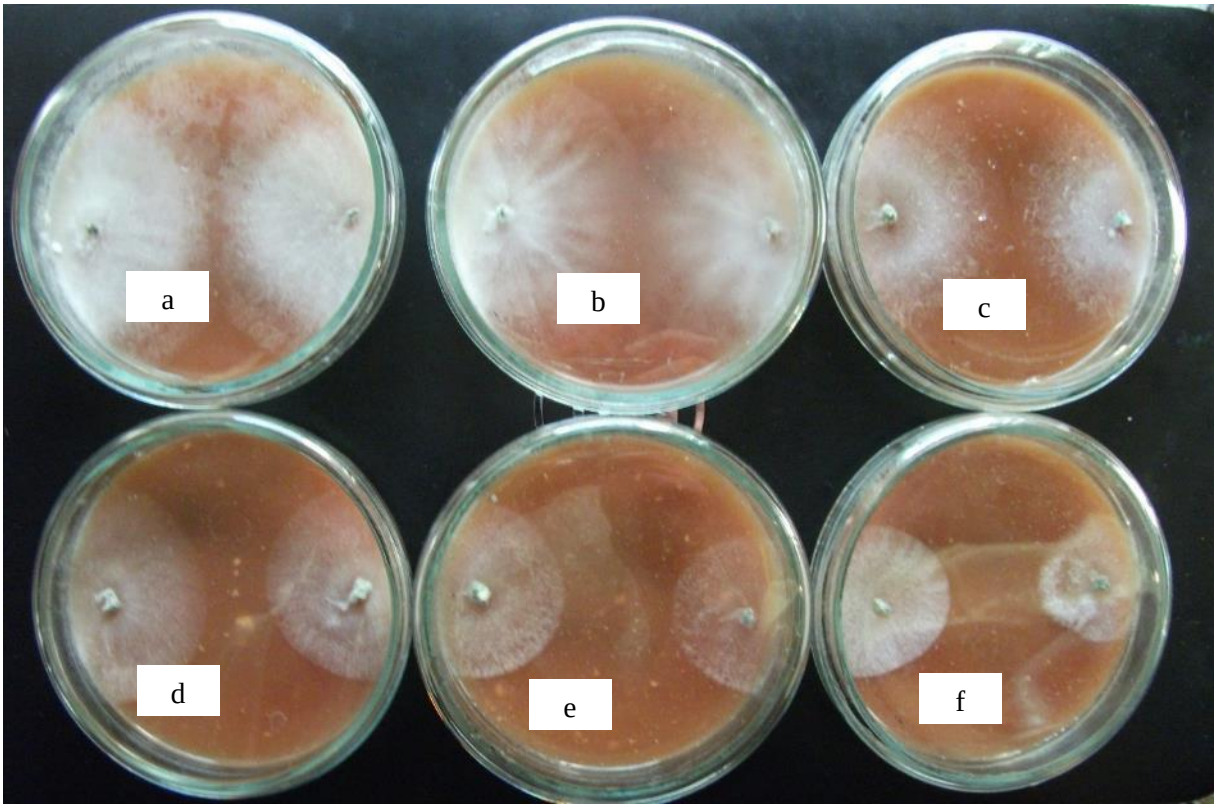

Figure 4-11. Results of *Trichoderma harzianum* ST4B cultivation of tomato pulp agar medium containing different concentration of untreated acidic whey after 4 days incubation at 25 ^{0}C. a: 0% v/v acidic whey, b: 20% v/v acidic whey, c: 40% v/v acidic whey, d: 60% v/v acidic whey, e: 80% v/v acidic whey, f: 100% v/v acidic whey.

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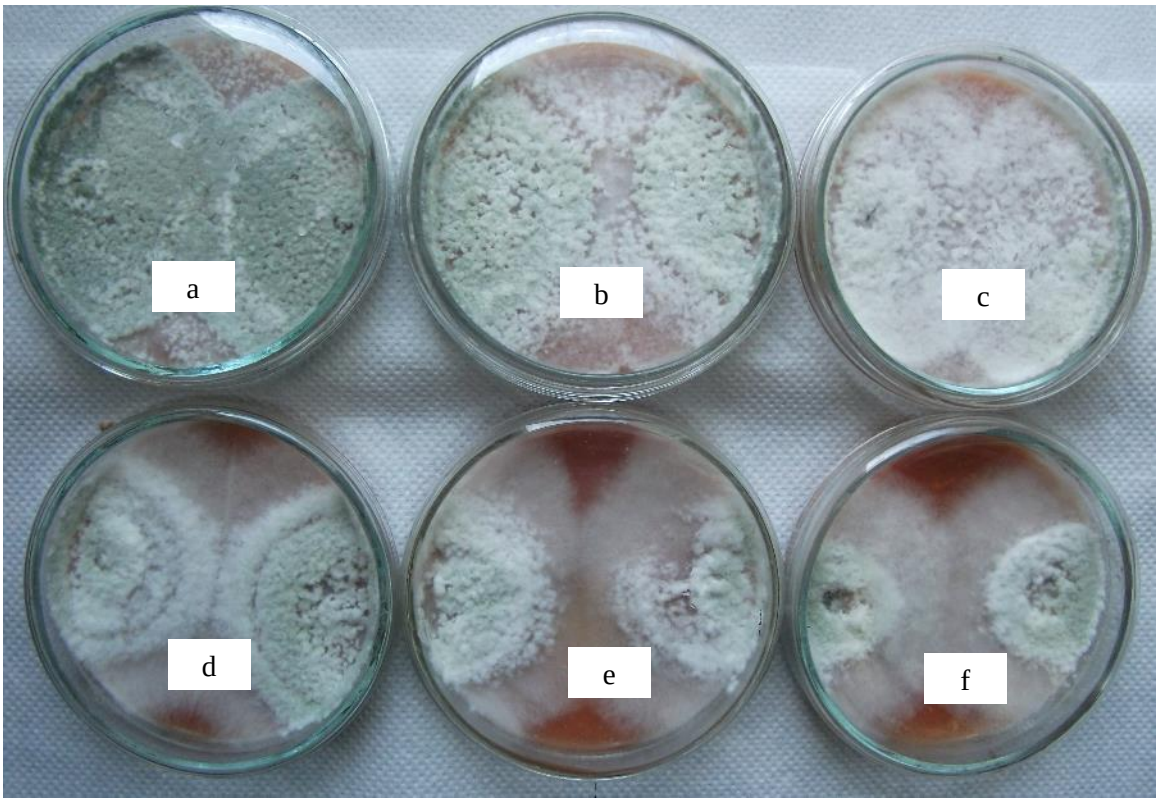

Figure 4-12. Results of *Trichoderma harzianum* ST4B cultivation of tomato pulp agar medium containing different concentration of untreated acidic whey after 7 days incubation at 25 ^{0}C. a: 0% v/v acidic whey, b: 20% v/v acidic whey, c: 40% v/v acidic whey, d: 60% v/v acidic whey, e: 80% v/v acidic whey, f: 100% v/v acidic whey.

In the third agar plate test, tomato pulp was added in six different concentrations (20, 40, 60, 80, 100 and 120 g/L) to the 100 %v/v acidic whey medium and inoculated with ST4B. The results of the measurement of the growth radius of fungal colonies are presented in Table 4-17, Table 4-18 and Figure 4-13.

The growth radius of *Trichoderma harzianum* ST4B fungal colonies was not significantly increased by the increasing concentration of tomato pulp and was lower compared to the tomato pulp agar control. **According to the results of the tomato pulp-acidic whey agar plate tests, the concentrated acidic whey waste water must be diluted in order to obtain a suitable liquid fermentation medium.**

Figure 4-14 shows the results of *Trichoderma harzianum* ST4B strain growing on tomato pulp- and tomato pulp-undiluted acidic whey agar medium contains 20-40-60-80-100 g/L tomato pulp after 4 days incubation at 25 °C. This figure is demonstrating the slower mycelial development in the case of tomato - acidic whey agar with 100% v/v acidic whey concentration compared to the tomato pulp agar medium. Addition of different quantities of tomato pulp did not compensate the inhibitory effect of the undiluted acidic whey.

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Table 4-17: The measured colony growth radius of Trichoderma strains ST4B strain on tomato pulp and tomato pulp-undiluted acidic whey (AW) agar medium containing 20-40-60-80-100-120 g/L tomato pulp after 2 days incubation at 25 °C.

	Tomato-pulp agar colony growth radius (cm)		AW + 20g tomato pulp agar colony growth radius (cm)		AW + 40g tomato pulp agar colony growth radius (cm)		AW + 60g tomato pulp agar colony growth radius (cm)		AW + 80g tomato pulp agar colony growth radius (cm)		AW + 100g tomato pulp agar colony growth radius (cm)		AW + 120g tomato pulp agar colony growth radius (cm)	
ST4B	2.9	2.5	0.5	0.5	0.6	0.6	1.0	1.0	1.2	1.0	1.2	1.0	1.2	1.0
ST4B	2.5	2.8	0.5	0.5	1.6	1.0	1.0	1.2	1.2	1.0	1.0	1.0	1.0	1.0
ST4B average	2.7		0.5		0.9		1.1		1.1.		1.1		1.1	

Table 4-18: The measured colony growth radius of Trichoderma strains ST4B strain on tomato pulp- and tomato pulp-undiluted acidic whey (AW) agar medium containing 20-40-60-80-100-120 g/L tomato pulp after 3 days incubation at 25 °C.

	Tomato-pulp agar colony growth radius (cm)		AW + 20g tomato pulp agar colony growth radius (cm)		AW + 40g tomato pulp agar colony growth radius (cm)		AW + 60g tomato pulp agar colony growth radius (cm)		AW + 80g tomato pulp agar colony growth radius (cm)		AW + 100g tomato pulp agar colony growth radius (cm)		AW + 120g tomato pulp agar colony growth radius (cm)	
ST4B	4.5	5.7	2.5	2.2	2.2	2.2	2.2	2.7	2.2	3.2	2.7	2.7	2.5	2.7
ST4B	4.7	5.2	2.0	2.1	3.2	2.6	3.1	2.7	2.7	2.2	2.8	2.8	2.7	2.0
ST4B average	5.0		2.2		2.1		2.7		2.6		2.8		2.5	

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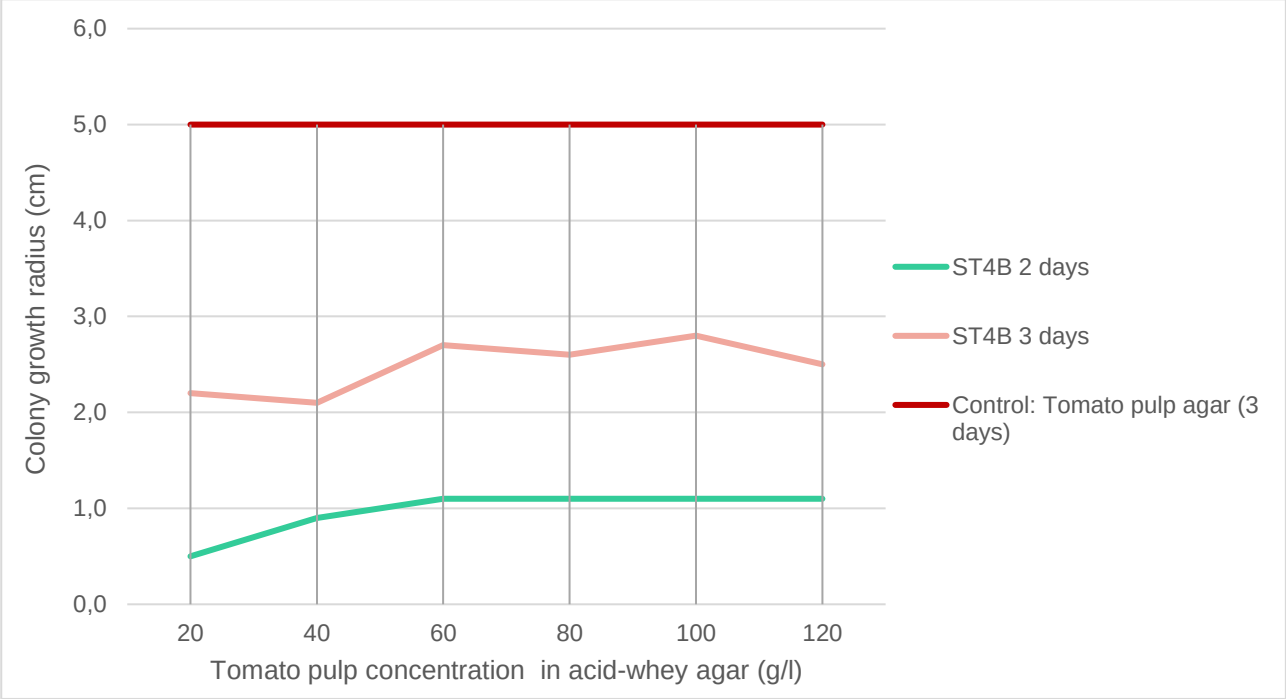

Figure 4-13. Changes in the colony growth radius of *Trichoderma harzianum* ST4B strain on tomato pulp – undiluted acidic whey (AW) agar medium containing 20-40-60-80-100 g/L tomato pulp after two- and three-days incubations period at 25 °C.

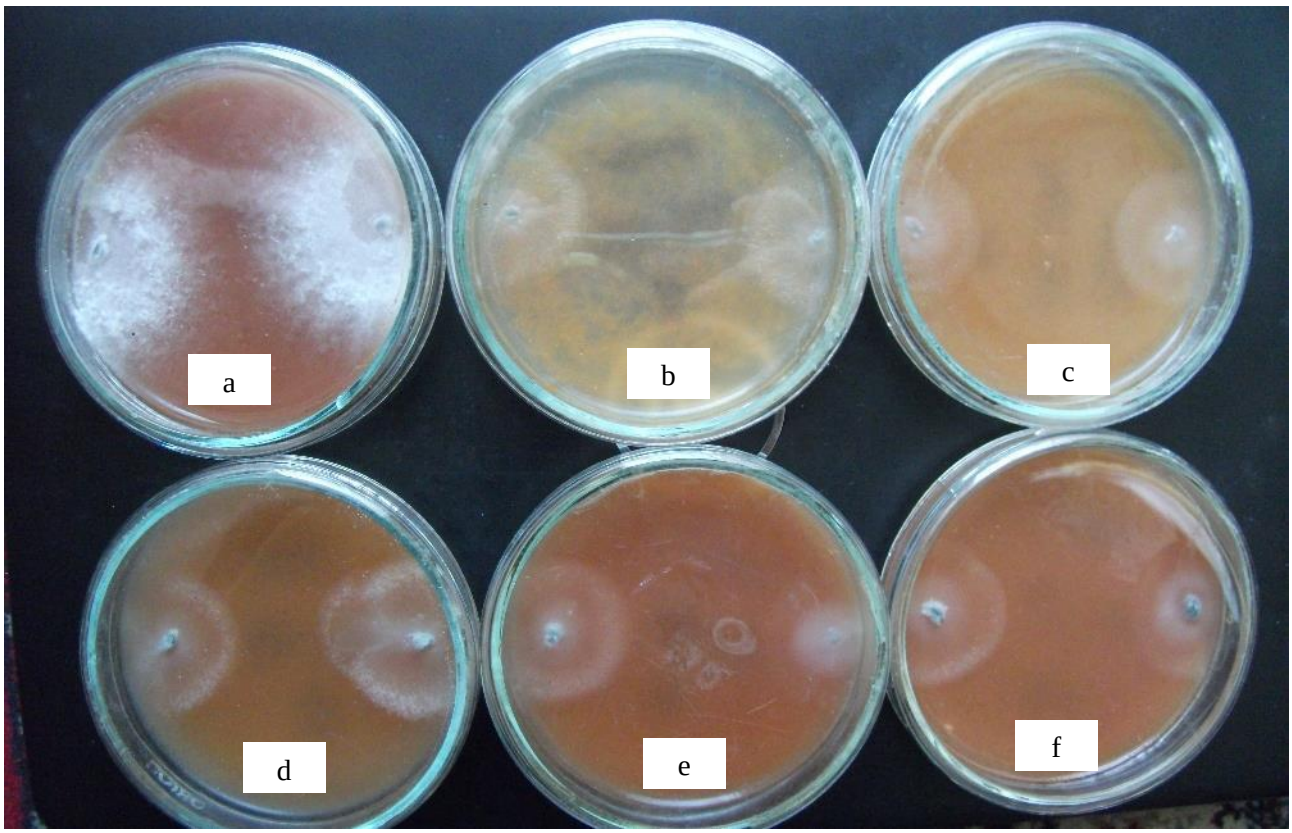

Figure 4-14. Results of *Trichoderma harzianum* ST4B strain on tomato pulp- and tomato pulp-undiluted acidic whey (AW) agar medium containing 20-40-60-80-100 g/L tomato after 4 days incubation at 25 °C. a: tomato-pulp agar, b:acidic whey – 20 g tomato pulp, c: acidic whey – 40 g tomato pulp, d: acidic whey – 60 g tomato pulp, e: acidic whey – 80 g tomato pulp, f: acidic whey – 100 g tomato pulp.

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4.2.2. Results of the shake flask culture tests for fermentation medium design

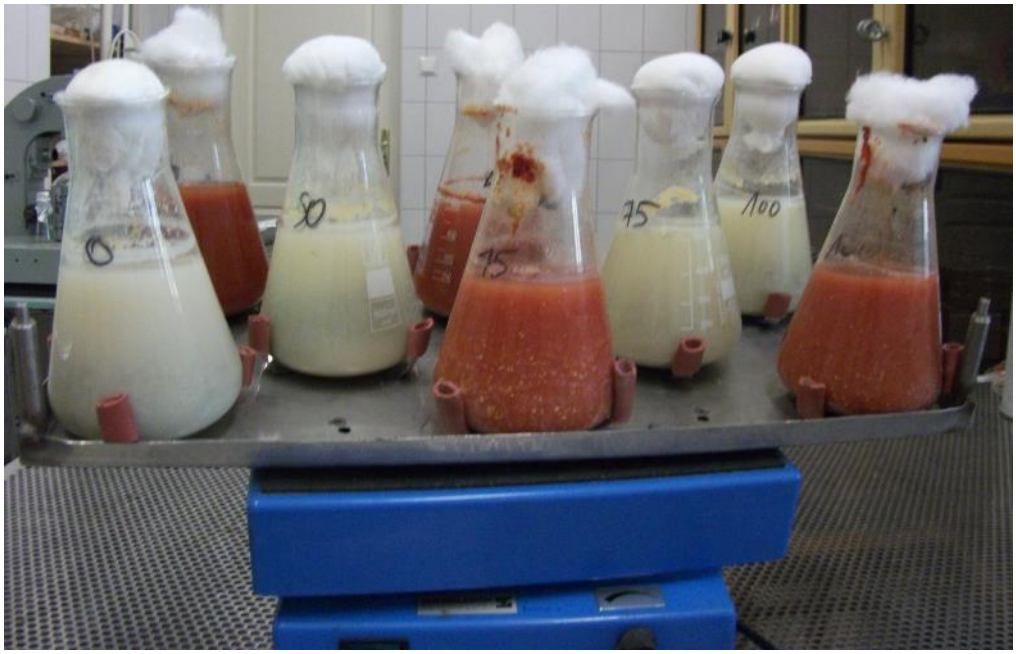

Figure 4-15. Shake flask culture test layout with different concentration of acidic whey (0-50-75-100% v/v) – tomato liquid media and acidic whey (0-50-75-100% v/v) – potato liquid medium for fermentation medium design after an incubation period of 7 days at 25°C.

Table 4-19: Results of the measured wet biomass weight and calculated dried biomass weight in shake flask cultures with different concentration of acidic whey (0-50-75-100% v/v) – tomato liquid media.

	Wet biomass weight (g)	**Dry matter content (%)**	**Calculated dried biomass (g)**
Tomato pulp – 0%V/V acidic whey	97.2	14.5	14.1
Tomato pulp – 50% V/V acidic whey	92.3	12.6	11.6
Tomato pulp – 75% V/V acidic whey	79.4	11.4	9.0
Tomato pulp – 100% V/V acidic whey	70.2	10.6	7.4

Table 4-19 shows the measured wet biomass weight and calculated dried biomass weight in shake flask cultures with different concentration of acidic whey (0-50-75-100% v/v) – tomato liquid media. **The highest biomass yield was achieved with the tomato pulp liquid media followed by tomato pulp – 50% v/v acidic whey liquid media.** The biomass yield decreased by the increasing concentration of the acidic whey.

Figure 4-16 shows the produced wet *Trichoderma harzianum* ST4B biomass in shake flasks contained different tomato pulp – acidic whey liquid medium after separation. The two samples (0% v/v acidic whey-tomato pulp and 50% v/v acidic whey-tomato pulp) had a similar appearance: bright red in colour and dense due to the intensive mycelial growth. The other two samples, which contained the acidic whey in higher (75 and 100% v/v) concentrations had lighter colour and higher moisture content.
The differences were also reflected in the filtering properties. **Separation of biomass from liquid medium contained 50% v/v and 0% v/v acidic whey were much easier than those containing higher concentrations of acidic whey (75-100% v/v).**

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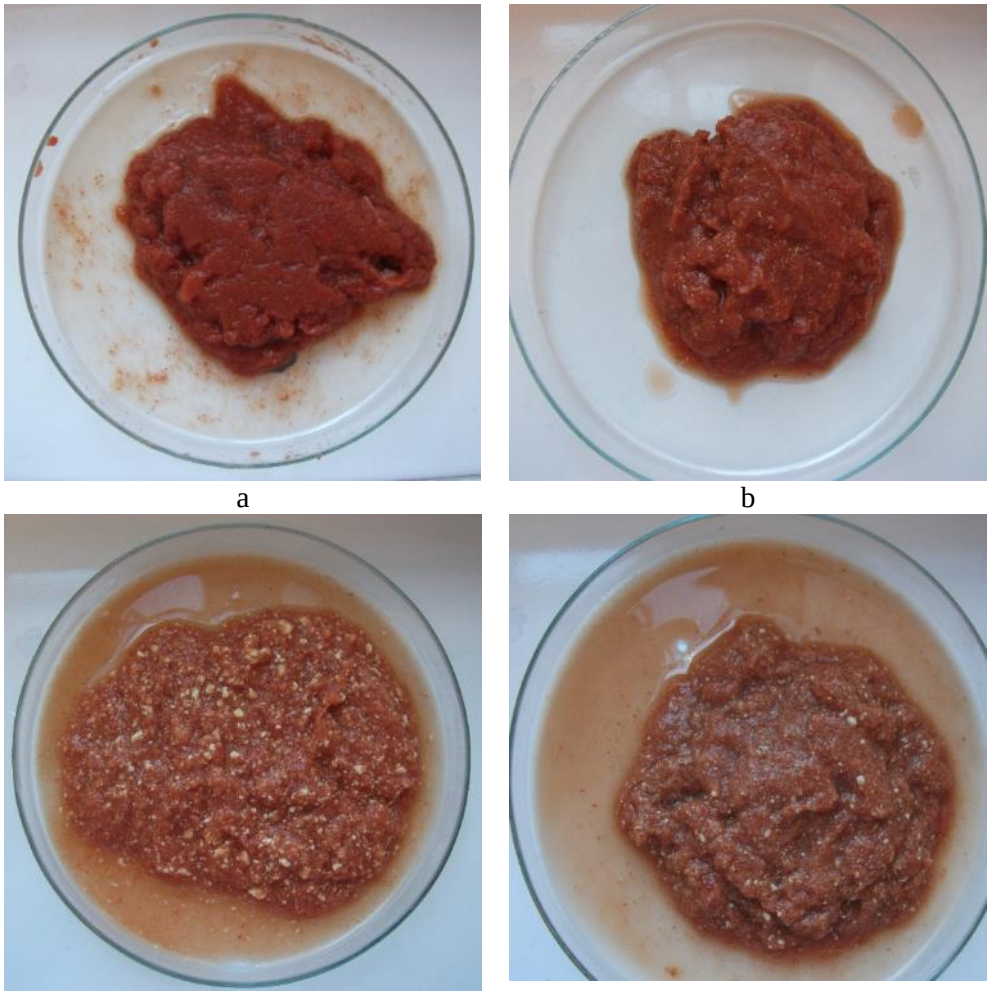

a b c d

Figure 4-16. The produced wet *Trichoderma harzianum* ST4B biomass from the different shake flasks contained different tomato pulp – acidic whey liquid medium after separation. Incubation period: 7 days at 25°C. a: tomato pulp – 0% v/v acidic whey medium, b: tomato pulp – 50% v/v acidic whey medium, c: tomato pulp – 75% v/v acidic whey medium, d: tomato pulp – 100% v/v acidic whey medium.

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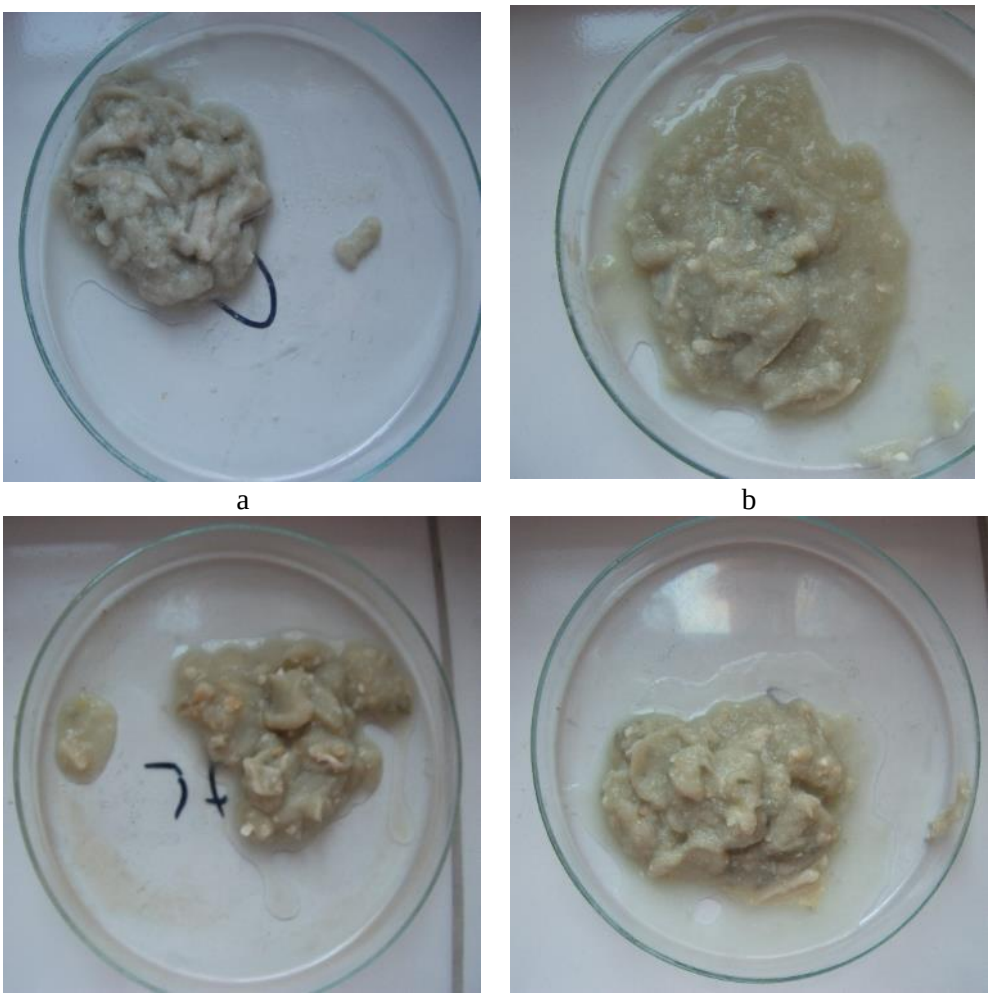
a b

Figure 4-17. Shake flask culture test with different concentration of acidic whey (0-50-75-100% v/v) – potato liquid medium for fermentation medium design. a: after inoculation with ST4B spore suspension, 0 day, b: after an incubation period of 7 days at 25 °C.



a b

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Figure 4-18. The produced wet *Trichoderma harzianum* ST4B biomass from the different shake flasks contained different potato – acidic whey liquid medium after separation. Incubation period: 7 days at 25°C. a: potato – 0% v/v acidic whey medium, b: potato – 50% v/v acidic whey medium, c: potato – 75% v/v acidic whey medium, d: potato – 100% v/v acidic whey medium.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement 101000752.

Figure 4-18 shows the wet *Trichoderma harzianum* ST4B biomass from the different shake flasks contained different potato – acidic whey liquid medium after separation. Compared to the tomato-acidic whey samples, the separation of biomass from the liquid medium was more difficult due to the less biomass yields.

Table 4-20: Results of the measured wet biomass weight and calculated dried biomass weight in shake flask cultures with different concentration of acidic whey (0-50-75-100 v/v%) – potato liquid media.

	Wet biomass weight (g)	**Dry matter content (%)**	**Calculated dried biomass (g)**
Tomato pulp – 0% v/v acidic whey	40.5	16.5	6.7
Tomato pulp – 50% v/v acidic whey	44.2	13.4	5.9
Tomato pulp – 75% v/v acidic whey	34.6	11.4	3.9
Tomato pulp – 100% v/v acidic whey	39.5	12.5	4.9

Table 4-20 shows the measured wet biomass weight and calculated dried biomass weight in shake flask cultures with different concentration of acidic whey (0-50-75-100% v/v) – potato liquid media. The highest biomass yield was achieved with the potato liquid media followed by potato – 50% v/v acidic whey liquid media. Less biomass yields were achieved from the potato-acidic whey liquid medium compared to the tomato pulp-acidic whey liquid medium.

The results of the shake flask test indicated that the optimal medium for scale up of the liquid fermentation is the tomato pulp-acidic whey with 50% v/v concentration.

4.2.3. Results of the laboratory scale validation of the liquid fermentation at 3x3 L scale.

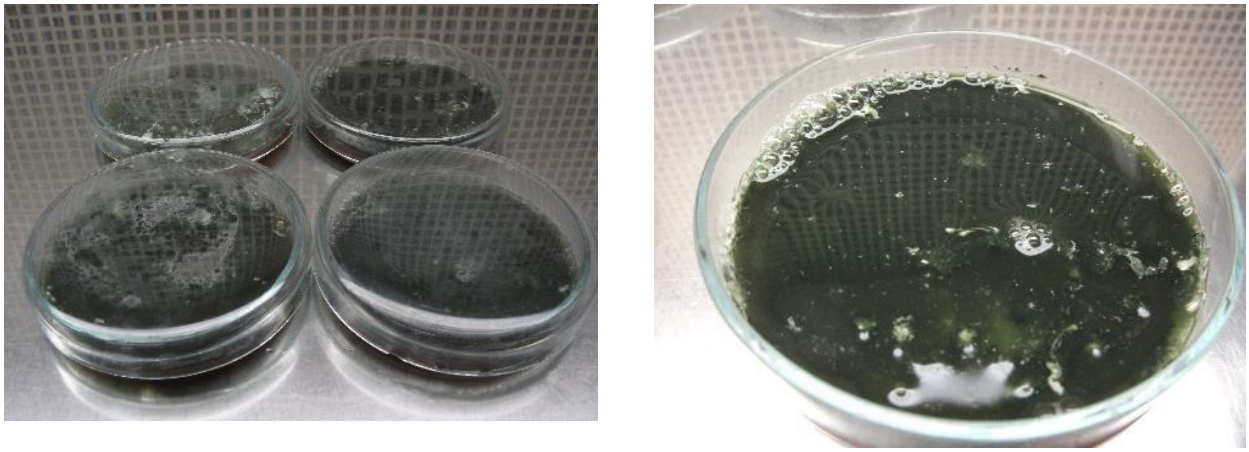

Figure 4-19. *Trichoderma harzianum* ST4B spore suspension for liquid fermenter inoculation.

Figure 4-20 and Figure 4-21 show the *Trichoderma harzianum* ST4B fungal biomass in the 3 L liquid fermenter after three days liquid fermentation on tomato pulp 50% v/v acidic whey medium. Figure 4-21 shows the result of the *Trichoderma harzianum* ST4B liquid fermentation after three days liquid fermentation on tomato pulp 75% v/v acidic whey medium.

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High yields of active fungal biomass were achieved on tomato pulp with 50% v/v acidic whey medium in the 3x3 L liquid fermenters system. The biomass was easily separated from the fermentation medium.

Trichoderma harzianum ST4B liquid fermentation on 75% v/v acidic whey was unsuccessful due to the inhibition effect of the acidic whey at higher concentration. A longer fermentation time might be more effective in the case of a higher acid whey concentration, however, this option was rejected for economic reasons for large scale fermentation process.

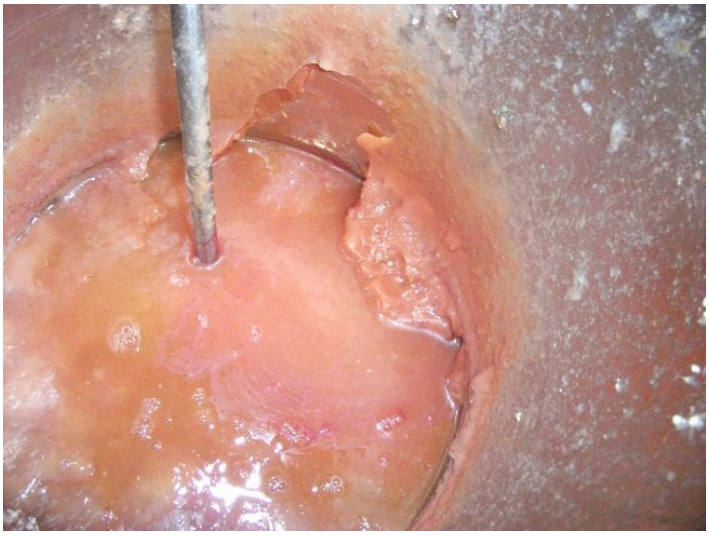

Figure 4-20. *Trichoderma harzianum* ST4B liquid fermentation after three days liquid fermentation on tomato pulp with 50% v/v acidic whey medium.

Figure 4-21. *Trichoderma harzianum* ST4B fungal biomass after three days liquid fermentation on tomato pulp with 50% v/v acidic whey medium.

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Figure 4-22. *Trichoderma harzianum* ST4B liquid fermentation after three days liquid fermentation on tomato pulp with 75% v/v acidic whey medium.

The liquid phase fermentation processes were successfully optimised for efficient vegetative cell (mycelia) production of the *Trichoderma harzianum* ST4B. During the optimisation process a very effective, simple and cost-efficient liquid fermentation process was developed. ST4B was easily fermented on tomato pulp-50 % v/v acidic whey liquid medium and high biomass volume was obtained which was in active mycelial growing phase that is essentially for the effective second stage solid fermentation.

Optimal fermentation conditions for Trichoderma ST4B fermentation on acidic whey:

- Sterile fermentation condition
- Agitation: only air stirred, mechanical mixing not used. Air flow rate: 0.2-0.5 L/min·L liquid
- pH: 4.5-5
- free sugar content: 2-3 %
- dry matter content: max. 5%
- temperature: 23-25 ºC
- Incubation period: 3 days

The optimal liquid medium composition was the tomato pulp with 50% v/v untreated acidic whey:

Tomato pulp (20%)	360 g
Glucose	24 g
Filtered tap water	1500 mL
Acidic whey	1500 mL

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4.2.4. Results of the laboratory scale tests for development of solid-state fermentation for BioPhosphate production

Inoculum: ready fermented Trichoderma from stage 1 3x3 liquid fermentation.

Table 4-21: Colony forming unit of the different samples

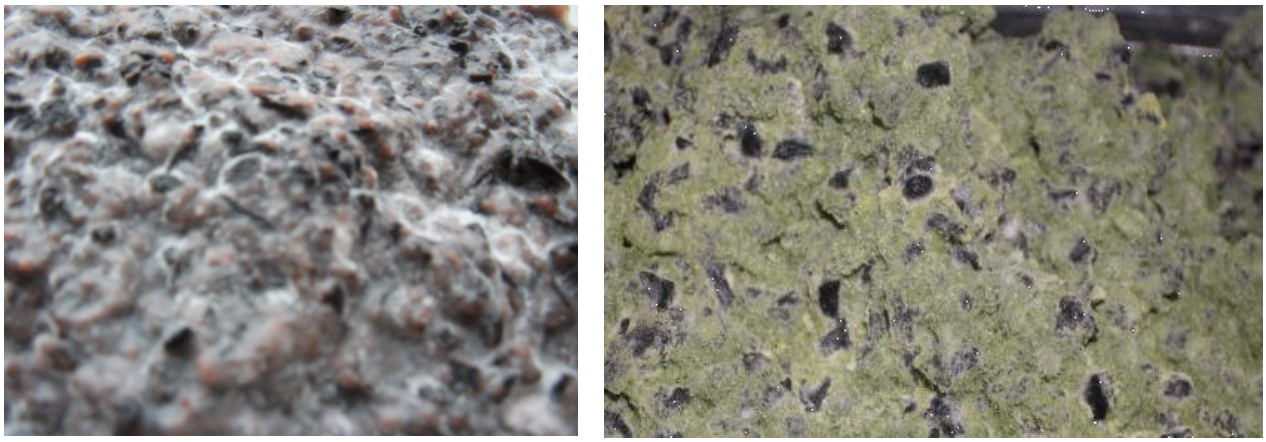
	CFU (Colony forming unit)
Test 1: Tomato pulp , 100 mL distilled water	$5.0 \cdot 10^6$
Test 2: Tomato pulp , 100 mL acidic whey	$3.5 \cdot 10^6$
Test 3: Tomato pulp , 50 mL distilled water, 50 mL acidic whey	$4.3 \cdot 10^6$
Test 4: Tomato pulp , 20 mL distilled water, 80 mL acidic whey	$3.9 \cdot 10^6$
Test 5: Tomato pulp , 80 mL distilled water, 10 mL acidic whey	$4.5 \cdot 10^6$

The high-quality inoculum obtained from the liquid fermentation contributed to successful colonisation of the animal bone char and production of BioPhosphate. The results (Table 4-20) of the CFU measurement show that there was no significant difference in CFU numbers for the different test conditions. During the solid fermentation, the added acidic whey did not affect the growth of the fungus.

The following solid state fermentation condition was successfully developed and applied for BioPhosphate production:

- semi-sterile condition (sterile: ABC and nutrient, air supply, semi-sterile: fermenter and sampling)
- Animal bone char
- Nutrient: sterile tomato pulp
- T=20ºC
- air supply: 2x15 min/day
- Agitation: not used
- Duration of the fermentation 7 days.

During the solid fermentation process semi-sterile condition was sufficient. Agitation was not used, because it has negative effect on the growth and conidia formation of *Trichoderma* spp. during the solid fermentation.



a b

Figure 4-23. *Trichoderma harzianum* ST4B mycelia growing on the animal bone char after 3 days (a) and 7 days (b) solid fermentation period.

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4.3. Results of the laboratory scale experiments for adsorption tests of the dairy WW stream acidic whey

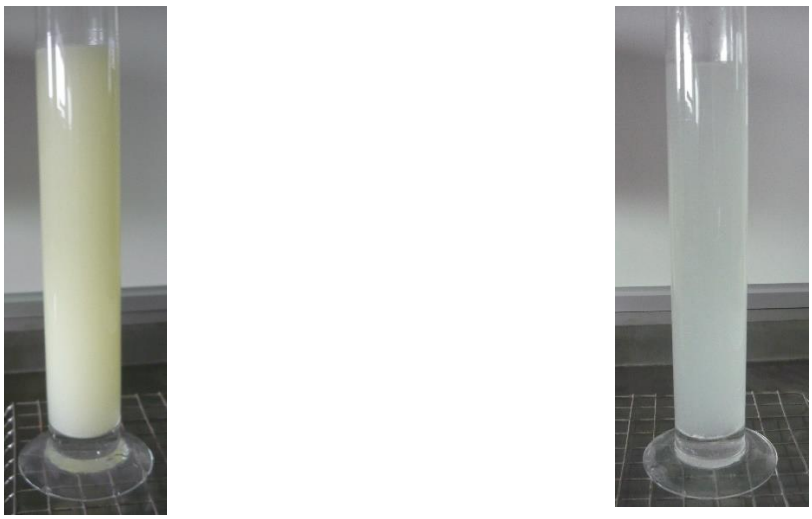
Adsorption treatment objectives of the dairy industrial WW adsorption:

1. Fully upcycle an unexploited dairy WW stream, e.g. high added value transformation of unexploited biomass into new products, perceived to be of greater quality and environmental/climate value with second life and new function that finished product becomes more practical and valuable than what it previously was.
2. Fully close the nutrient cycle for dairy food processing.
3. Implement economically viable full circularity through the full dairy food industrial processing chain that is applicable towards high TRL targets as well.
4. Reduce pollution, energy use and greenhouse gas emissions, where energy neutrality and climate neutrality are in focus.
5. Improve the water quality by adsorption technique that makes it viable for reuse of effluent water to close the nutrient cycle for regional dairy farmer operations.

As pharmaceuticals are not target contamination in the dairy food industrial WW acidic whey stream, this effluent meets all the EU WW effluent standards and the next decades of updated WW regulation standards as well for long term. The EU is continuously updating WW regulations and aims to achieve stricter WW regulations by 2040 and a pollution-free environment by 2050.

Two types of liquid streams processed, such as:

a) Direct adsorption processing of the dairy WW stream acidic whey and
b) Adsorption processing of the separated fermentation liquid residuals.



Before ABC treatment After ABC treatment

Figure 4-24 Result of the adsorption test

Adsorption test parameter results:

- The macroporous ABC adsorbent mass ratio is 625 g/L that adsorbed 760 mL liquid solution.
- The equilibration time for adsorption is up to 5 minutes and the amount of test substance food industrial WW acidic whey with pH4 and 93% water content adsorbed at equilibrium.
- The low pH is adjusted and neutralised by the ABC high Ca content in combination with food grade sodium bicarbonate powder water soluble additive.
- The adsorption of the acidic whey on the surfaces of the ABC and the stability of the test substance during the test period is successfully demonstrated.
- The adsorption on the ABC is studied at a single concentration as the food industrial WW acidic whey concentration and composition is rather similar and not changing.

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Table 4-22: Results for direct adsorption processing of the dairy WW stream acidic whey.

Target contamination (case a)	Input stream	Output effluent	Targeted
pH	4.54	7.00	7.00
Total suspended solids mg/L	1080	35	35
COD mg/L O_2	66800	3760	25
BOD	17500	1627	125
Total P mg/L	837	116	2
Total N	686	19	10

Table 4-23: Results for adsorption processing of the separated fermentation liquid residuals.

Target contamination (case b)	Input stream	Output effluent	Targeted
pH	4.54	7.00	7.00
Total suspended solids mg/L	62	32	35
COD mg/L O_2	135	21	25
BOD	105	111	125
Total P mg/L	21	2	2
Total N	36	8	10

The results indicate for both cases that by adsorption technique upcycling and reuse of the treated effluents for moisturising irrigation clean water at the targeted composting applications is possible. Water and the main treatment line fermented output biofertilisers can be delivered back to regional dairy farmer's location to co-compost cattle manure and close the nutrient cycle. In this context, full circularity and zero emission target, dairy WW collection, treatment, monitoring and effluent reuse are fully implemented inside the sector. The biotech fermentation upcycling process is considered as the main treatment line and the adsorption processing is a supportive process to close the nutrient and water cycle.

Consideration for the adsorption of the other food industrial WWs:

Beyond the main target of the dairy WW acidic whey stream processing, the corn milling, brewery and soft drinks, olive milling and winery WW streams are also small lab scale adsorption tested. The results:

a) Corn milling WW: the ABC adsorption of corn steep liquor liquid by-products is not possible as the high load of organic parts provides external coating on the adsorber. Furthermore, the industry already solved the full recovery, closed loop recycling and onsite reuse of process WWs by separating liquids from solids, cleaning and feed back to the main process. The industry is already using process water cleaning and

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exclusive blends of custom-designed WW treatment formulations for closed loops recycling and process reuse of WW, therefore it is expected that no added value can be offered by WalNUT for this case.

b) Brewery/soft drinks WW: The sanitation cleaning WW steam closed loop recovery and reuse of grey water is already solved locally at each factory and not suitable for nutrient recovery new business model.
c) Olive milling WW: the ABC adsorption of olive milling liquid by-products is not possible as the high load of phenol organic compounds provides external coating on the adsorber that makes the ABC not suitable for this type of treatment.
d) Winery WW: the sanitation cleaning WW steam closed loop recovery and reuse of grey water is already solved locally at each winery and not suitable for nutrient recovery new business model.

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5. Next steps

Based on the previous results 3R is developing, designing, implementing and testing a TRL5 WP3 pilot liquid and solid-state fermentation technology where the food industrial waste water WW acidic whey will be used as liquid medium during the fermentation process. Acidic whey WW is the overwhelming largest liquid by-product stream of the dairy food industry with significant economic importance. This problematic liquid stream is converted into useful biofertiliser with high nutrient recovery efficiency while environmental impacts minimised. The liquid fermenter will be inoculated by selected agriculturally beneficial microbial strains (*Trichoderma* spp.) that are selected specifically for the acidic whey biotech processing, consisting high lactose content and low pH4. Additional food industrial by-products will also be added to the liquid medium. The liquid fermenter will be inoculated by a selected agriculturally beneficial microbial strains (*Trichoderma* spp.) that are selected specifically for the acidic whey biotech processing, consisting high lactose content and low pH4. Unique macroporous adsorbent applied (ABC BioPhosphate) that is a high P and Ca density material, which is REACH tested as of T4.5. that is the regulatory precondition of WP3 TRL5 and WP4 demo operations at <10 t/y scale. The problematic liquid stream is converted into P/Ca based multi-functional compound biofertiliser with combined effects by multi-stage processing for wide range of product functional applications (as of safety, quality and labelling compliance defined by the (EU) 2019/1009 PFC categories) and high nutrient recovery efficiency while environmental impacts minimised. The high nutrient density compound BBF BIO-NPK-C output products delivered to regional users, who will use it for their onsite CMC3 green compost material enrichment and improvement where the technological irrigation water is also reused. The circular economy based 3R provides biotech upcycling solution aiming the nutrient recovery from the unexploited main waste water stream dairy food industrial acidic whey, which problematic WW is transformed into resource and converted into market competitive multifunctional biofertiliser production for sustainable reuse to close the nutrient loop.

Table 5-1: Next steps: timing and objectives

WP	Timing	Objectives
2	M1-M18	Characterise, design and test dairy industrial acidic whey WW upcycling technology for NR at lab scale, while considering technical, economic and environmental aspects. Concept development of the liquid/solid state fermentation and integrated ABC-BioPhosphate adsorption technology of food industrial WW streams. D2.5.
3	M14-M38 1) M14-M18 design 3R TRL5 pilot plant. Contract for TRL5 equipment building and installation. 1) M19-M24 build and install the 3R TRL5 pilot plant equipment. 2) M25-M26 start-up of 3R TRL5 pilot plant. 3) M27-M38 operation and optimisation of 3R TRL5 pilot plant to produce enough BBFs to perform field trial.	Demonstration and validation of TRL5 pilot at relevant scale (D3.4). Lawful validation of TRL5 pilot for REACH 1-10 t/y legal conformity assessment and certification completed before M19 action start (ECHA registration number: 01-2119490075-38-0042). All to keep the tight WP3 time schedule, subtask 3.1.3 pre-operation follow up CAD design completed January end 2023 and TRL5 equipment building and installation manufacturing contracted February end that is to be completed before M24 at the latest. Multi-functionality safety, quality and labelling legal compliance as of (EU) 2019/1009 PFC categories to be completed before M38. **P1-Product**: Fungus formulated ABC BBF. **P2-Product**: Nutrient formulated multifunctional adsorbent ABC BBF.
4	M12-M54 1) M12-M18 assessment of quality requirements and	Demonstration and validation of biofertilisers at relevant scale (D4.5, D4.6 and contribution to other deliverables)

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	EU regulation conformity, including REACH 2) M19-M24 assessment and preparation of demo action 3) M25-M54 demonstration and validation of BBF at relevant scale and identification of application practices	
5	**M10-M54**	Impact assessment
6	**M1-M54**	Policy implications at regional/national and European level (Bring knowledge from WP1-5. collaborates with WP7, WP8)
7	**M12-M54**	Integrated business model and full value chain
8	**M1-M54**	Communication, Dissemination and Community Management

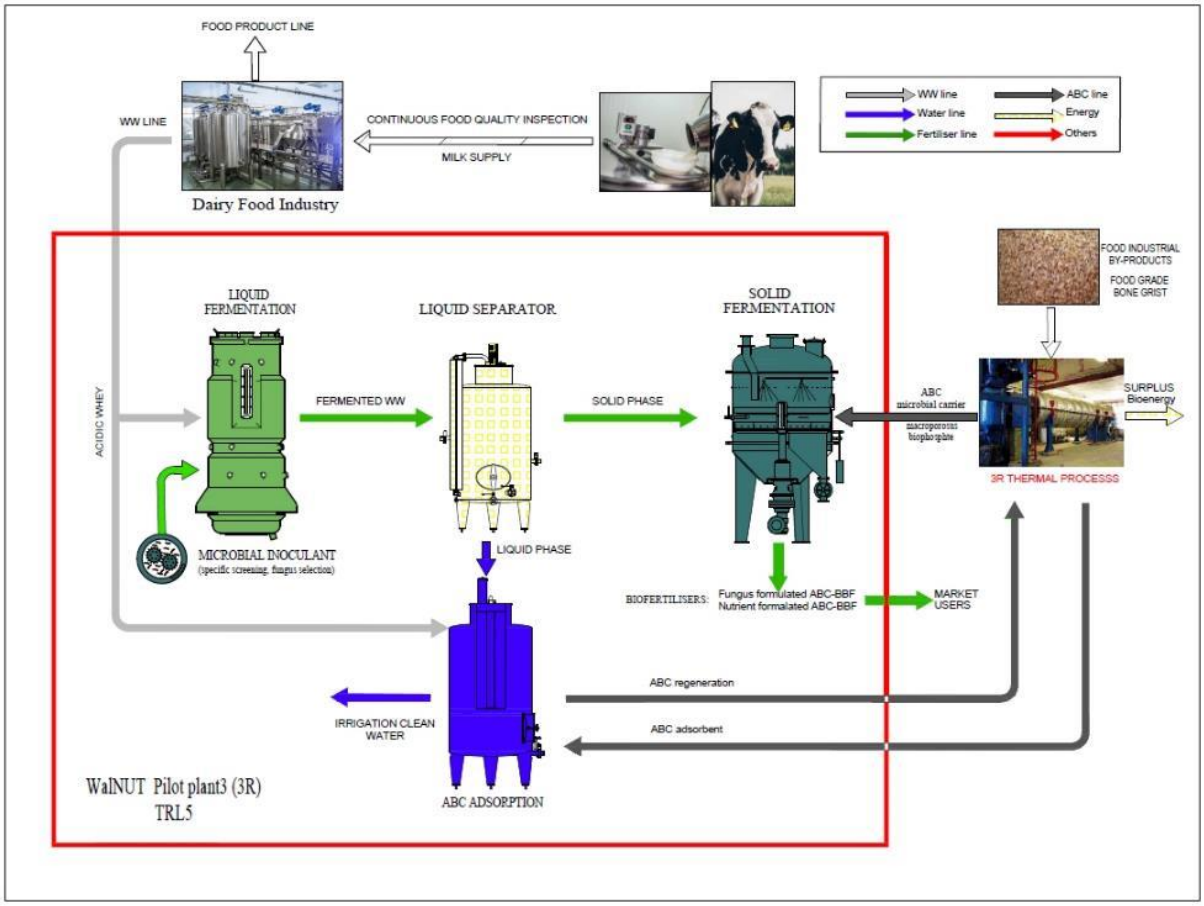

Figure 5-1. Flow diagram of integrated 3R up-cycling of food industrial waste water and by-products for production of clean water and economically high concentrated biofertilisers at less cost.

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Additional food industrial by-products will also be added to the liquid medium. The liquid fermenter will be inoculated by a selected agriculturally beneficial microbial strains (*Trichoderma* spp.) that are selected specifically for the acidic whey biotech processing, consisting high lactose content and low pH4. The BIO-NPK-C compound BBF is humus co-composted where the technological irrigation clean water is also reused for moisture control. In this context a community of organisms fostered that by use decompose organic material and break down matter, improve soil structure, and create a prolific soil ecosystem and fertile environment for healthy plant growth. The lawful final product quality and safety will be checked. The 3R **WP3 TRL5 pilot validation scales:**

1) 150 L/batch liquid fermenter.
2) 500 L/batch solid fermenter.
3) Liquid separator to separate the fermented WW liquid/microbiologically concentrated solid parts.
4) Adsorber equipment.

Adsorber to treat the fermented liquid parts or directly the acidic whey. ABC adsorbent will be used for final treatment of the separated liquid effluent from the liquid fermentation process. The end-product is a clean irrigation water for horticultural application or other industrial uses.

Unique macroporous adsorbent applied (ABC Animal Bone Char or BioPhosphate) that is a high P (35% P_2O_5) and Ca (37% CaO) density material, which is REACH tested and validated as of T4.5 that is the regulatory precondition of WP3 TRL5 and WP4 demo operations at <10 t/y scale.

In this context the core problem of the dairy sector is with the 90% WW stream acidic whey, which is industrially unsolved and therefore consisting WalNUT focus and objective driven strategy, such as the "nutrient recovery from waste water", re-design the value and supply chains of nutrients" and "to showcase the full potential of waste water as a raw material for biofertilisers production". Acidic whey WW is the overwhelming largest liquid by-product stream of the dairy food industry with significant economic importance. This problematic liquid stream is converted into biofertiliser with high nutrient recovery efficiency while environmental impacts minimised. In this context the 3R provides biotech solution for the nutrient recovery from the main waste water WW stream acidic whey produced by the dairy food industry that is a recovered resource raw material. The total dairy WW acidic whey material treatment volume is 120 m^3.

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6. Conclusions

The dairy food industrial WW stream acidic whey is the overwhelming largest and problematic liquid by-product stream of the dairy food industry with significant economic importance that is unexploited, consisting of 93% water content and characterised by extreme high BOD/COD/TOC and as low pH as 4.5. The WalNUT project development is upcycling this problematic liquid WW stream with high nutrient recovery efficiency and converting into P/Ca based multi-functional compound biofertiliser with combined effects by multi-stage processing for wide range of product functional applications. The safety, quality and labelling compliance defined by the (EU) 2019/1009 PFC categories and REACH while environmental impacts minimised.

ABC animal bone char is highly optimal and efficient to adsorb macromolecular organic contamination in liquid stream and adjust pH. The ABC animal bone char adsorbent is recently REACH registered and certified by the EU ECHA Authority 1-10 t/y that is the regulatory precondition for lawful TRL5 implementations. The combination of fungus biotech fermentation main process with adsorption processing is suitable for WalNUT added value new nutrient recovery business model with "Blue Ocean" Strategy.

In this context the problematic WW is transformed into resource and converted into market competitive multifunctional biofertiliser production for sustainable reuse to close the nutrient loop for the dairy farmers and food processing industry. The reduction of pollution, energy use and greenhouse gas emissions targeted, aiming to fully close the dairy industry nutrient cycle for dairy food processing where energy- and climate neutrality are in focus.

The high nutrient density compound BBF BIO-NPK-C output products delivered to regional users, who will use it for their onsite CMC3 green compost material enrichment and improvement where the technological irrigation water effluent is also reused.

The dairy industry nutrient cycle for carbon, nitrogen, phosphorus and water is developed, which aims to compensate the exit of mineral elements that are necessary for animals and plants that is an essential contribution to the circular economy. Closing those cycles improving self-sufficiency of dairy farms. Tackling the existing nutrient flow gaps in Europe will help decrease greenhouse gas emissions, reduce soil degradation and improve EU independence for energy, nutrients and water supply to dairy farm operations, such as irrigation water to moist on-farm composting.

The concept of the ABC-BioPhosphate: multi-functional biofertiliser and adsorbent that has been successfully developed. The liquid/solid state fermentation and integrated ABC-BioPhosphate adsorption technology of food industrial dairy waste WW stream (acidic whey) were successfully validated at laboratory scale at petri dish agar culture, 500 mL shake flask cultures and 3x3 litre fermentation for low-cost production of recovered BBFs and to obtain a high-quality final effluent up-cycled clean water. The 3R successfully developed, designed and tested a specific liquid and solid-state fermentation technology with interconnected adsorber where the food industrial WW was used as liquid medium during the fermentation process.

After selection procedure and biotech optimisation, finally three different *Trichoderma harzianum* strains (STA, ST4B, ST5) with origin from soil and rhizosphere of the 3R strain collection were used for the selection of suitable strains capable of growing on untreated acidic whey food industrial waste water. The results of the acidic-whey agar culture medium tests showed that all the three (STA, ST4B, ST5) *Trichoderma harzianum* strains were able to grow on acidic whey substrate and could utilise the lactose as carbon source possible due to the lactase enzyme activity of Trichoderma fungus.

However, in the case of acidic whey a slower growth rate and longer sporulation time of *Trichoderma harzianum* strains were observed compared to the tomato pulp agar as a control growing medium. In the case of using tomato pulp-acidic whey growing medium the increasing concentration of the acidic whey content caused an increased inhibitory effect of the acidic whey compared to the growing medium contains tomato pulp only. The laboratory scale results indicated that concentrated acidic whey waste water must be diluted and combined with other substrate in order to obtain a suitable liquid fermentation medium.

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For developing the efficient fermentation nutrient strategy we **successfully combined acidic whey in 50% v/v with other tomato pulp and liquid fermentation** that scaled up made to 3x3 L capacity.

In the laboratory scale solid state fermentation tests the separated biomass from the 3x3 L liquid fermenter was used. **The second solid state fermentation condition was successfully developed** for production of biotech formulated BioPhosphate.

More efficient and sustainable farm business models created for dairy food industrial nutrient recovery for the interest and benefits of dairy farmers and dairy food industrial processors.

1) The combination of biotech fermentation main process with adsorption processing of the separated fermentation liquid residuals, reuse of irrigation effluent to moisturise co-compost cattle manure and close the nutrient cycle is the most **efficient and viable solution for efficient upcycling of the unexploited dairy industrial WW acidic whey** where the full circularity and zero emission targets implemented.
2) The combination of biotech fermentation main process with adsorption processing of the separated fermentation liquid residuals is **suitable for WalNUT added value nutrient recovery new business model.**
3) **The ABC animal bone char adsorber with high macro-porosity is highly optimal substance** to specifically treat the unexploited dairy WW streams with macromolecular contaminations.
4) The ABC animal bone char adsorber is recently REACH validated, registered and certified by the EU ECHA Authority 1-10 t/y (no. 01-2119490075-38-0042) and ready for lawful WP3 and WP4 TRL5 implementations.
5) The adsorption treatment objectives of the dairy industrial WW adsorption in the case of combination of biotech fermentation main process with adsorption processing of the separated fermentation liquid residuals and reuse of effluents are fully achieved.
6) The biotech fermentation upcycling process is considered as the main treatment line and the adsorption processing is a support to complete and close the nutrient and water cycle for the dairy farmers and processors.
7) The direct adsorption processing of the dairy WW stream acidic whey is a technical possibility, but the input target contamination is too high to make cost efficient one step treatment.
8) The other food industrial WW adsorption lines from corn milling, brewery/soft drinks, olive milling and winery sectors are technically not suitable for efficient nutrient recovery and economical cleaning with the ABC adsorbent, also it has been recognised that these sectors already solved the recycling and reuse of grey water onsite at each factory and not suitable for WalNUT added value nutrient recovery new business model.

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