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Interconnection of Waste Chicken Feather Biodegradation and Keratinase and *mcl*-PHA Production Employing *Pseudomonas putida* KT2440

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Abstract

Background and objective: Waste chicken feather is an important waste product of the poultry processing industry and is annually produced in substantial amounts. Hence, wise management of this waste is desirable. In this work we aimed at feathers biodegradation by a selected bacterial strain capable of utilizing chicken feathers as sole carbon source-*Pseudomonas putida* KT2440. To utilize feather, the bacterial culture excrete keratinase, which can be easily isolated after biodegradation process and which, therefore, represents an interesting side product of the intended technology. Moreover, bacterial culture of employed for feather degradation is also capable of *mcl*-PHA accumulation.

Materials and methods: Bacterial culture of *Pseudomonas putida* KT2440 was cultivated in presence of waste chicken feathers as the only carbon source; during the cultivation keratinase activity and biomass growth were monitored. Metabolically active biomass after feather degradation was used for *mcl*-PHA production.

Results and conclusion: During cultivation on waste feathers, bacteria did not accumulate detectable amounts of medium-chain length polyhydroxyalkanoate (*mcl*-PHA); nevertheless, when metabolically active bacterial cells after feather biodegradation were transferred into nitrogen limited mineral media, a high medium-chain length polyhydroxyalkanoate content of 61% of cell dry weight in microbial cells was reached. The polymer consisted of 3-hydroxyhexanoate (27.2% mol) and 3-hydroxyoctanoate (72.8% mol) monomer units. Therefore, this work demonstrates a possible interconnection of feather biodegradation with keratinase and medium-chain length polyhydroxyalkanoate production.

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1. Introduction

Millions of tons of chicken feathers are generated as waste by the poultry processing industry every year, which represents serious problem for the food industry. According to Taskin et al. poultry processing manufac-turers produce about 7.7×10^8 kg of feathers annually [1]. Nevertheless, despite low prize and abundant availability of feathers, its further applications as well as valorization approaches are limited. Waste feathers is used in low-grade animal feed but more often is treated as a waste and ends up in landfills or incinerators. These ways of destroying feathers are not desirable from an economical as well as an ecological point of view and they have been restricted or even banned in some countries [2]. Therefore; there is an urgent need for new processes enabling ecological and economical valorization of this resistant solid waste. There are some studies aimed at utilization of waste chicken feathers as a substrate for processes of microbial biotech-nology. Taskin et al. [3] reported that chicken feathers protein hydrolysate can be used for biotechnological manufacturing of L-lactic acid by *Rhizopus oryzae*; waste chicken feathers can be also utilized for production of biogas, xylanase or extracellular polysaccharides [4-6]. Recently, we have reported that alkaline hydrolysate of chicken waste feathers can be applied as inexpensive complex nitrogen source to enhance *scl*-PHA production on waste frying oil employing *Cupravidus necator* [7].

Feathers consist predominantly (about 90% weight) of proteins, the most important fraction is represented by keratin. Feathers keratins are small fibrous proteins with a molecular weight around 10 kDa, which are of interest due to their high nutrition value caused by a desirable amino acids composition. On the other side, keratin is mechanically and chemically robust and also resistant to either chemical or enzymatic hydrolysis, which complicates its digestibility and applicability for feeding purposes [8]. Efficient enzymatic hydrolysis of keratin is possible only by action of specific proteolytic enzymes called keratin-ases. These enzymes are capable of complete hydrolysis of feathers and other keratin rich substrates. Therefore, these enzymes have numerous applications in food and feed industry, fertilizer production, cosmetics or pharmaceutical and textile industries [9].

Keratinases for industrial purposes are usually produced by microorganisms especially bacteria belonging to genus Bacillus. For instance Williams et al. isolated, identified and characterized feathers degrading bacteria *Bacillus* (*B.*) *licheniformis* [10]. Actually, all the currently commercially available preparations of keratinases are produced by *B. licheniformis* [9]. Furthermore; there are numerous recent reports that also members of the genus Pseudomonas are efficient producers of keratinases. Mohamad et al. isolated *Pseudomonas* sp. LM10 which was capable of providing high keratinase activities in feathers based media supplemented with fructose and peptone [11]. Similarly, Stiborova et al. isolated *Pseudomonas* sp. P5 demonstrating substantial feathers hydrolyzing capability [2]. Keratinase production was also recorded for *Pseudomonas* (*P.*) *aeruginosa* [12].

In addition, numerous Pseudomonas strains are also capable of accumulating polyhydroxyalkanoates (PHAs) as carbon and energy storage materials. These microbial polyesters of hydroxyacids are considered an ecological alternative to petrochemical plastics since they are fully biodegradable, biocompatible and moreover they are produced from renewable sources [13]. PHAs are usually accumulated by bacterial cells when a carbon source is present in excess but other elements such as nitrogen or phosphorous are exhausted. Depending on the monomer composition, PHA are classified as *scl*-PHA (monomers consist of 3-5 carbon atoms) and medium-chain length polyhydroxyalkanoate (*mcl*-PHA) (monomers contain 6-14 carbon atoms). Most Pseudomonas strains are producers of *mcl*-PHA [13]. Among all the Pseudomonads, *P. putid*a KT2440 is the most studied in the context of PHA metabolism and is the most widely used for *mcl*-PHA production [14-16]. The strain (originally designated as *P. arvilla* strain mt-2 and subsequently reclassified as *P. putida* mt-2) is a plasmid-free derivative of a toluene-degrading bacterium. It is considered being probably the most-studied saprophytic laboratory Pseudomonad which is able to survive and prosper in the environment [17]. Therefore, apart from its PHA production capabilities, it is also candidate strain for industrially-oriented metabolic engineering aimed at various applications [18].

Aware of the interesting PHA production capacities of numerous Pseudomonads and their capability of utilization of waste feathers which is associated with keratinase excretion we tested numerous Pseudomonas strains for their feathers biodegrading capacities. Our intent was to interconnect highly interesting process of feathers degradation with *mcl*-PHA as well as keratinase production. Surprisingly, we observed that *P. putida* KT2440 revealed the highest degradation efficiency and keratinase activity in cultivation media when waste chicken feathers was used as a sole carbon source in cultivation media. Therefore, we employed this well-known *mcl*-PHA producing bacterial strain for development of our concept interconnecting keratinase and *mcl*-PHA production.

2. Materials and methods

2.1. Microorganism and cultivation method

P. putida KT2440 (DSM 6125) was purchased from Leibnitz Institute DSMZ-German Collection of Microorganism and Cell Cultures, Braunschweig, Germany.

Non-treated chicken feathers were used as sole carbon source for growth of the bacteria culture and keratinase production. The cultivation was carried out for 7 days at 30°C with shaking at 180 rpm in 100 ml mineral salt medium (MSM) containing (g l^{-1}) $(NH_4)_2SO_4$, 1; KH_2PO_4, 1.02; Na_2HPO_4, 12; H_2O, 11.1; $MgSO_4$ $7H_2O$, 0.2. The MSM included 1 ml l^{-1} microelements solution (MES) which contains (g l^{-1}) $FeCl_3$ $6H_2O$, 9.7; $CaCl_2$ $2H_2O$, 7.8; $CuSO_4$ $5H_2O$, 0.156; $CoCl_2$ $6H_2O$, 0.119; $NiCl_2$ $6H_2O$, 0.118; $ZnSO_4$ $7H_2O$, 0.1; dissolve in 0.1 M HCl. First, the feathers were washed and dried and after being sterilized (121°C, 30 min.) they were used as a sole carbon source in an amount corresponding to 20 g per liter of MSM. The flasks were inoculated with an overnight culture of *P. putida* grown in Nutrient Broth medium which consist of (g l^{-1}) bacteriological peptone, 10; beef extract, 10; and NaCl, 5. Total loss of feathers, biomass, pH and keratinase activity was determined during the cultivation, all the cultivations were performed in duplicate.

Bacterial biomass obtained after feathers degradation was separated from feather by filtration (Whatman filters), centrifuged (5000 ×g, 5 min) and re-suspended in production medium consisting of (g l^{-1}) $(NH_4)_2SO_4$, 1 or 0; KH_2PO_4, 1.02; Na_2HPO_4 $12H_2O$, 11.1; $MgSO_4 \cdot 7H_2O$, 0.2 and 1 ml l^{-1} MES. Further, the cultivation medium was supplemented by waste frying oil (oil was obtained from university canteen at FCH BUT, it consisted predominantly of rapeseed oil) (20 g l^{-1}) and octanoic acid (2 g l^{-1}) which served as the carbon sources and *mcl*-PHA precursors, the cultivations were performed in duplicate.

2.2. Analytical methods

Keratinase activity of the supernatant was determined and expressed as reported by Letourneau et al. using Keratinazure (Sigma-Aldrich) as a substrate [19].

Biomass concentration was measured gravimetrically as cell dry mass (CDM). PHA content in bacterial cells was analyzed employing GC-FID as reported previously, particularly 3-hydroxyalkanoic acid (C6, C8, C10, C12 and C14) purchased from Larodan (Sweden) were used as a standards, benzoic acid was used as internal standard [20].

Elemental composition of the chicken feathers (obtained from poultry processing manufactory Vodnanska drubez a.s., Brno Modrice, Czech Republic) before and after degradation was determined using a CHNS/O analyzer Euro Vector EA 3000. Samples (~ 1-2 mg) were packed in tin capsules in the oven for combustion at 980°C using pure oxygen as combustion gas and pure helium as carrier gas. All elements were determined by thermal conductivity detector (TCD). The calibration curves for C, H, N, S were obtained using a reference standard sample sulphanil-amide.

FTIR-ATR spectra of feather before and after degradation were obtained by means of an Attenuated Total Reflectance (ATR) technique using a Nicolet iS50 spectrometer. All spectra were recorded over the range 4000-400 cm^{-1} at a resolution of 4 cm^{-1} and were the average of 256 scans.

3. Results and discussion

We screened various Pseudomonas strains originating both from culture collections as well as our isolates for waste chicken feathers degradation capability which is associated with keratinase production. Surprisingly, among all the tested strains, *P. putida* KT2440 demonstrated the highest chicken feather degradation capacity. When the bacterial strain was inoculated into the mineral media containing waste chicken feathers as the sole carbon source and cultivated under optimal conditions, the process of feathers biodegradation was visible to the naked eye (see Fig. 1) solid particles of waste chicken feathers were transformed into pulp-like material, which was accompanied by decrease of weight of the feathers and also by substantial increase of turbidity of the cultivation media. When the cultivation was finished, it was possible to remove partially degraded chicken feathers by filtration using Whatman no. 5 filter paper. Not surprisingly, bacterial cells were not captured by the filter paper and passed to the filtrate. Therefore, it was possible to separate bacterial cells for further purposes and gravimetrically quantify both measure of feathers degradation and concentration of bacterial biomass.

Furthermore, we investigated the time course of feathers degradation, keratinase production and biomass growth during an experiment lasting for 7 days, the results are demonstrated in Fig. 2. As expected, the growth of the bacterial culture on feathers was accompanied by secretion of keratinase into the cultivation media. The highest biomass concentration was achieved on the 5th day of cultivation (1.06 g l^{-1}). The keratinase activity in cultivation media rapidly increased, reaching the maximal level (approx. 13 U ml^{-1}) on the 2nd day of cultivation and remained constant till the 5th day of cultivation. After that, keratinase activity in the cultivation media decreased to approximately 8 U ml^{-1}. It is likely that the factor which inhibited growth of the bacterial culture and decreased keratinase excretion was the high pH value, since the pH value of the cultivation media increased during the time course of cultivation which is very typical feature when protein rich substrates are utilized as the carbon source by microbial culture which is accompanied by excretion of basic nitrogen-containing metabolites into cultivation media. pH value reached its maximal value 8.6 on the 5th day of cultivation. Nevertheless, despite the decrease in metabolic activity of the bacterial culture and keratinase activity in cultivation media after the 5th day of cultivation, chicken feather degradation progressed almost linearly during the whole assay. After 7 days of cultivation 28% of feathers' weight was completely degraded. It is likely that the measure of feather degradation could have been enhanced by prolongation of the biodegradation process or even by control of pH in the cultivation media.

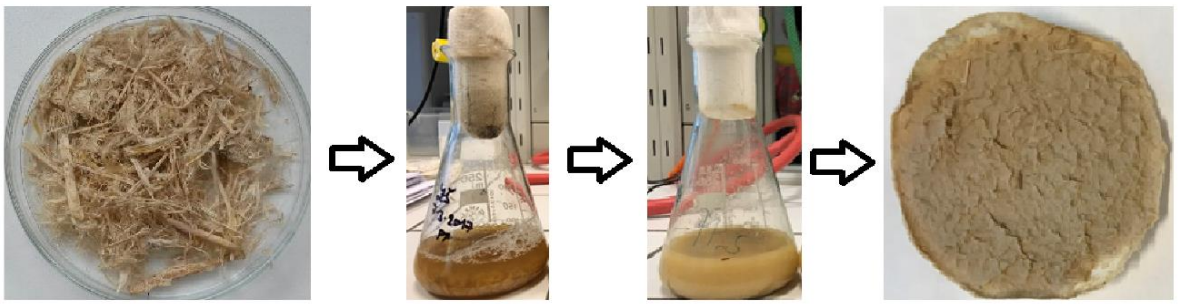

Figure 1. Demonstration of feather degradation by *Pseudomomas putida* KT2440 during 7 days. Large solid particles of chicken feather were degraded by microbial culture resulting in formation of pulp-like material which can be removed by filtration using Whatman no.5 filter paper.

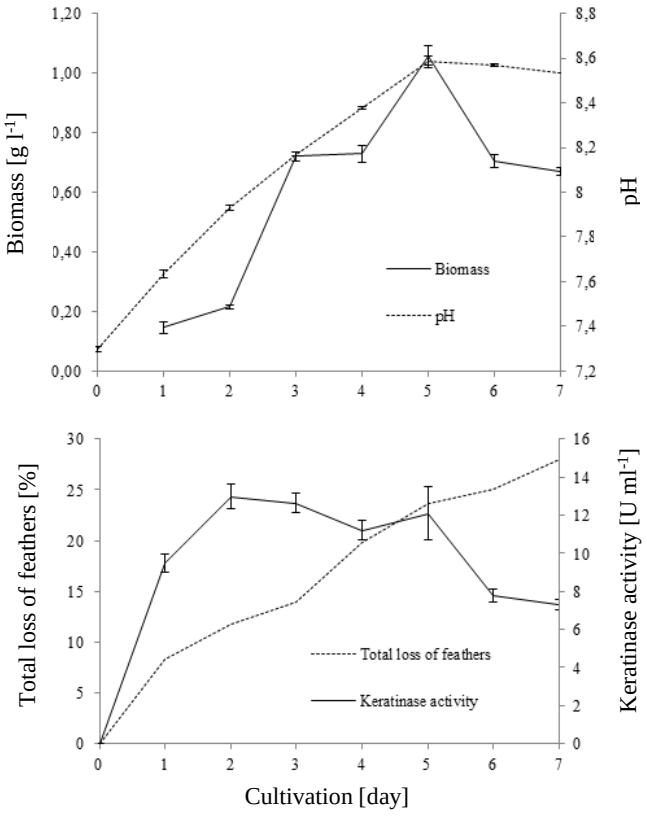

Figure 2. Time course of a) biomass growth and pH development b) keratinase activity and feather degradation expressed as percentage mass loss during 7 days lasting biodegradation experiment employing *Pseudomomas putida*.

It should be noted that the samples of bacterial biomass taken during feathers biodegradation assay were analyzed to determine the intracellular content of PHA. Never-theless, since PHA accumulation is activated by nitrogen limitation and feathers are rich in protein and therefore also in nitrogen content, we did not detect any PHA in bacterial biomass [21,22].

Further, we decided to investigate chemical changes of feathers induced by the degradation process. The materials before and after degradation were analyzed in terms of chemical composition of macro-elements using a combusting elementary analyzer and also by FTIR. The FTIR-ATR spectra of feathers before and after degradation are presented in Fig. 3. All the spectra contain a sharp and intensive band at 1629 cm^{-1} corresponding to carbonyl groups in amide I, an α-helix conformation. Another band occurring at 1520 cm^{-1} can be assigned to stretching C-N and N-H bending groups in amide II conformation, which are also indicated by the broad band centered at about 3272 cm^{-1} resulting from the N-H stretching vibrations of the amide II groups. The absorbance bands of aliphatic groups is revealed in the range 2980-2840 cm^{-1}, where the asymmetric and symmetric C-H stretching of methyl groups at 2961 cm^{-1} and 2875 cm^{-1}, and the asymmetric C-H stretching of methylene groups at 2927 cm^{-1}.

The deformation bending of methyl and methylene groups occur in the spectra at 1448 cm^{-1} and 1384 cm^{-1}. The less intensive absorption band at 1340 cm^{-1} corresponds with amide III. The appearance of amide III is revealed by 1330-1200 cm^{-1} frequency zones, where the N-H in-plane bending and C-N stretching vibrations in amide groups and corresponds to the α-helix conformation. The absorption band at 1070 cm^{-1} is characteristic a peak for skeletal C-C vibration. Finally, the appearance of the band at 1158 cm^{-1} is most probably associated with the C-C stretching in amino acids. The appearance and absorption bands of FTIR-ATR spectra suggest that the chemical composition of chicken feathers during degradation process was not substantially changed. Further, the elemental compositions of feathers before and after degradation are presented in Table 1. The results show that the average elemental compositions of feathers before and after degradation are comparable. Therefore, based on results of FTIR elemental analysis, it can be stated that degradation of chicken feathers by *P. putida* KT2440 is not associated with chemical modification of the solid material, but the process of feathers degradation consists of keratinase catalyzed cleavage of peptide bounds in feathers keratin. Decrease of molecular weight of keratin is most likely responsible for change of appearance and structure of the material (see Fig. 1, feathers before and after degradation).

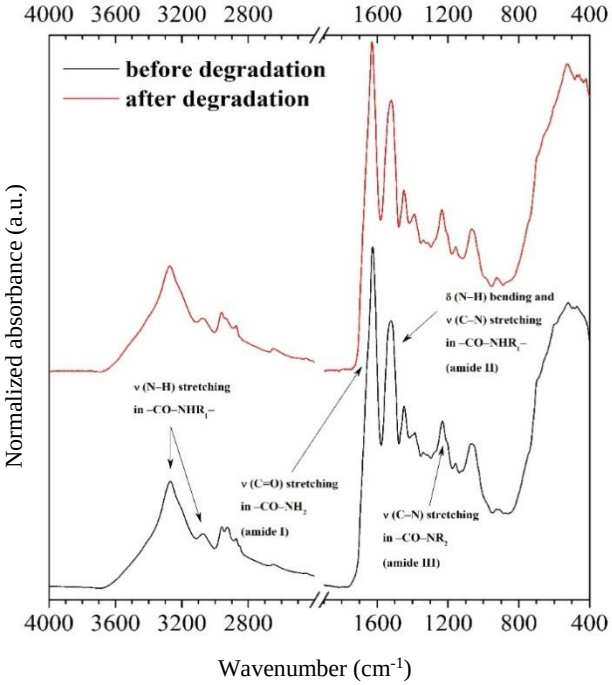

Figure 3. FTIR spectra of chicken feather before and after degradation

Table 1. Element composition of feather before and after degradation (7 days) by *Pseudomomas putida*

Feathers	Nitrogen (%wt)	Carbon (%wt)	Hydrogen (%wt)	Sulphur (%wt)
Before degradation	17.19 ± 0.30	53.15 ± 0.30	7.75 ± 0.13	0.59 ± 0.84
After degradation	16.37 ± 0.07	50.75 ± 0.04	7.63 ± 0.05	Not detected

Thus, it can be stated that several interesting side products are formed when *P. putida* is cultivated on waste chicken feathers. The first interesting product is the solid fraction remaining after fermentation- partially hydrolyzed feathers. This side product can be simply separated by filtration and it has potential use in animal feeding since partial hydrolysis of feathers keratin substantially improves its digestibility. For instance, Chayatip et al. used waste chicken feathers partially digested by *B. subtilis* to improve protein content of corncob silage [23]. Nevertheless, it should be noted that a thorough evaluation may be necessary before a potential use of the partially hydrolyzed feathers as an animal feed, in order to meet legal requirements. This may include the nutritional value of this product as well as determining the safety issue. The positive aspect of intended process is GRAS (generally recognized as safe) status of *P. putida* KT2440 [24].

Filtrate after residual feathers removal by filtration can be further separated by centrifugation. The supernatant contains substantial amounts of active keratinase, which can be also considered being interesting product finding application at various industrial fields [4,25]. Hence, this is the first report suggesting *P. putida* KT2440 as promising candidate for keratinase production. Nevertheless, further experiments are needed to investigate basic characteristics of the keratinase and to evaluate its usability in various applications.

Last but not least, the sediment after filtrate centrifugation contains metabolically active cells of *P. putida*. As was mentioned above, the bacterial culture did not accumulate PHA during feathers degradation since nitrogen limitation was not induced. Nevertheless, metabolically active biomass after feathers degradation can be simply employed for *mcl*-PHA production. Therefore, we re-suspended bacterial cells after feathers degradation in two formulations of mineral media- with and without 1 g l^{-1} of $(NH_4)_2SO_4$, sodium octanoate and waste frying oil were used as *mcl*-PHA precursor. PHA producing capacity of bacterial cells after feathers degradation was compared with control cultivation which was "traditionally" inoculated from overnight culture cultivated in NB medium, the results are demonstrated in Table 2.

During initial 24 h of cultivation, bacteria did not accumulate *mcl*-PHA regardless of presence or absence of nitrogen or bacterial cells origin. Nevertheless, when bacterial cells from feathers degradation were used, a higher initial amount of cells was introduced into the process (initial CDM was 0.15 and 0.7 g l^{-1} for control and biomass stemming from feather biodegradation respective-ly), which decreased consumption of carbon substrate for cell growth. After the first day of cultivation, bacterial cells started to accumulate *mcl*-PHA consisting exclusively of C6 and C8 monomers.

Table 2. Production of *mcl*-PHA by *Pseudomonas putida* cultures stemming from feather degradation process

		$(NH_4)_2SO_4$ in media	CDM (g l^{-1})	PHA (%wt)	PHA (g l^{-1})	C6 (%mol)	C8 (%mol)
24 h	Control	Yes	0.46 ± 0.08	*n.d.*	*n.d.*	-	-
	Cells from feather degradation	Yes	1.29 ± 0.22	*n.d.*	*n.d.*	-	-
	Cells from feather degradation	None	1.25 ± 0.15	*n.d.*	*n.d.*	-	-
48 h	Control	Yes	1.45 ± 0.11	61.5 ± 4.31	0.89 ± 0.09	44.0	56.0
	Cells from feather degradation	Yes	2.34 ± 0.12	49.3 ± 6.58	1.15 ± 0.16	9.7	90.3
	Cells from feather degradation	None	2.60 ± 0.15	48.7 ± 4.31	1.26 ± 0.13	28.5	71.5
72 h	Control	Yes	1.37 ± 0.10	42.3 ± 3.10	0.58 ± 0.06	61.0	39.0
	Cells from feather degradation	Yes	1.95 ± 0.18	26.1 ± 2.57	0.51 ± 0.07	*n.d.*	100.0
	Cells from feather degradation	None	2.31 ± 0.30	61.4 ± 8.27	1.42 ± 0.27	27.2	72.8

CDM stands for cell dry mass, *n.d.:* not detected, results are in form: mean ± standard deviation

It should be noted that cells from feather degradation supplied with nitrogen produced almost exclusively poly(3-hydroxyoctanoate), while complete nitrogen limitation resulted in presence of about 30% mol of 3-hydroxyhexanoate in the copolymer structure. Actually, 3-hydroxyoctoyl-CoA is preferred substrate for PHA synthase of *P. putida*; therefore, it is not surprising that the major constituent of *mcl*-PHA was 3-hydroxyoctanoate when the cells were cultivated in presence of octanoate [26]. The fact that strong nitrogen limitation increased the proportion of 3-

hydroxyhexanoate could be explained by the fact that nitrogen limitation stimulated action of enzymes which are responsible for conversion of S-3-hydroxacyl-CoA (stemming from β-oxidation of fatty acids) to R-3-hydroxy-acyl-CoA which can serve as a substrates for PHA synthase. Such an effect was recently reported by Mozejko-Ciesielska et al. [27] who observed that expression of the all the enzymes involved in *mcl*-PHA biosynthesis in *P. putida* is up-regulated under nitrogen limitation. The highest PHA yields were observed in the bacterial culture originally used for feathers degradation which was cultivated in complete absence of nitrogen. In this case, bacterial biomass contained 61.4% wt of *mcl*-PHA per cell dry weight and *mcl*-PHA titers reached 1.42 g l^{-1} which is about 2.4 fold more than yields in control cultivation. It is very likely that absence of nitrogen in the cultivation media strongly stimulated PHA accumulation. On the other side, this cultivation strategy cannot be applied in a traditional cultivation scenario in which presence of nitrogen for growth of biomass is a necessary condition to reach reasonable biomass and *mcl*-PHA yields.

4. Conclusion

To sum up, the suggested process, which is schematically depicted in Fig. 4, advantageously intercom-nects waste feathers degradation enabling biomass growth and *mcl*-PHA accumulation, which can be performed in a separate step in complete absence of nitrogen directing the substrate very efficiently to the *mcl*-PHA biosynthetic pathway. The *mcl*-PHA content in bacterial cells can, therefore, reach very high values which is beneficial not only from the perspective of overall productivity, but high PHA content in biomass has also a positive impact on the cost of the isolation process [28]. Moreover, keratinase and partially digested chicken feathers are very interesting side products of the suggested process, which can be used for instance in the food and feed industry.

5. Acknowledgements

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6. Conflict of interest

All the authors declare that they have no conflict of interest.

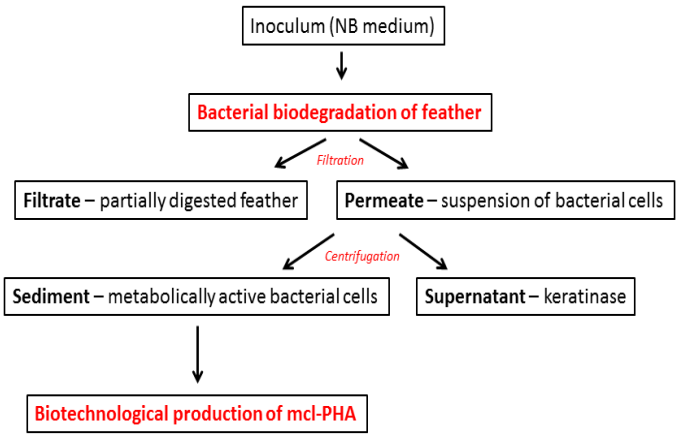

Figure 4. Idea of interconnection of feather biodegradation and *mcl*-PHA production

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ارتباط زیست تجزیه پذیری ضایعات پر مرغ و تولید کراتیناز و *mcl*-PHA با استفاده از *سودوموناس پوتیدا* KT2440

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چکیده

سابقه و هدف: ضایعات پر مرغ یکی از ضایعات مهم صنعت فرآوری طیور است که سالانه به میزان قابل توجهی تولید می‌شود. لذا مدیریت عالمانه این ضایعات مطلوب است. در این کار، هدف ما تجزیه زیستی پر توسط سوش باکتریایی منتخب *سودوموناس پوتیدا KT2440* بود که قادر به استفاده از پر مرغ به عنوان تنها منبع کربن می‌باشد.

مواد و روش‌ها: برای تجزیه پر از کشت باکتریایی *سودوموناس پوتیدا KT2440* که قادر به تجمع *mcl*-PHA می‌باشد استفاده شده و توانایی ارتباط تجزیه پر و تولید *mcl*-PHA بررسی شد.

یافته‌ها و نتیجه‌گیری: هنگام کشت پر، باکتری‌ها مقادیر قابل اندازه‌گیری *mcl*-PHA تولید نکردند؛ با این حال، پس از تجزیه پر، هنگامی که باکتری‌های فعال متابولیکی به محیط کشت با محدودیت نیتروژن منتقل شدند میزان تولید *mcl*-PHA افزایش و به ۶۰ درصد وزن خشک سلولی رسید. بسپار موجود شامل واحدهای مونومر ۳-هیدروکسی‌هگزانوآت (۲۷/۲ درصد مولی) و ۳- هیدروکسی اوکتانوآت (۷۲/۸ درصد مولی) بود. لذا، این کار امکان ارتباط تجزیه زیستی پر با کراتیناز و تولید *mcl*-PHA را نشان می‌دهد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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- ضایعات پر
- *سودوموناس پوتیدا*
- کراتیناز
- *mcl*-PHA

***نویسنده مسئول**

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