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SHORT COMMUNICATION

Production of polyhidroxyalkanoates by *Pseudomonas putida* KT2442 harboring pSK2665 in wa stewater from olive oil mills (alpechín)

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The nutritionally versatile *Pseudomonas putida* has showed to grow in olive oil waste water called "alpechín", which is toxic for many other microorganisms. The transformation with the plasmid pSK2665, harboring *Alcaligenes eutrophus* genes needed for synthesis of poly (3-hidroxybutyric acid), allow *Pseudomonas* strain to grow in high concentration of the wastewaters "alpechín" accumulating biodegradable thermoplastic.

Polyhydroxyalkanoic acid (PHA) is a biodegradable polymer material that accumulates in numerous microorganisms under unbalanced growth conditions as a mechanism of storing excess carbon and energy. These polymers are synthesized by the enzyme PHA synthase, which in bacteria is bound to the surface of the PHA granules and utilizes the coenzyme A thioesters of hydroxyalkanoic acids as substrates.

The poly (3-hidroxybutyric acid) (PHB) biosynthetic genes phbA (for 3-ketothiolase), phbB (NADPH-dependent acetoacetyl-CoA reductase), and phbC (PHB synthase) from acetyl-CoA have been cloned recently. These genes are clustered and are presumably organized in one operon. The genes have been expressed in *Escherichia coli* (Slater et al. 1988) and in different species of the genus *Pseudomonas* belonging to rRNA homology group I; they conferred the ability to accumulate polyesters consisting of 3-hydroxybutyrate on most of the recombinant cells (Timm et al. 1990).

PHB is a thermoplastic with many desirable properties because it is biodegradable and the current market demand for a biodegradable thermoplastic is enormous (Lafferty et al. 1988). It is also immunologically compatible with human tissue. *Alcaligenes eutrophus* is now used for commercial PHA production, but many other microorganisms

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accumulate PHB and can grow on more or different carbon sources than *A. eutophus*. (Page, 1992). Nevertheless, the much higher price of PHA compared with conventional plastic material has limited its current applications.

Olive oil extraction produces a large amounts of residues, the olive-mill waste waters are known as "alpechín". They are regarded as a severe environmental problem because of their high organic content, large simple phenolic compounds that are both antimicrobial and phytotoxic. One of the solutions for the bioremediation of alpechín is biopolymeric substance production from the residue, focusing on polysaccharides and biodegradable plastics production (Ramos-Cormenzana et al. 1995).

We describe a strain of *Pseudomonas putida* capable of accumulating PHA in "alpechín" media which may allow the development of novel culture methods for biodegradable microbial polyesters production from residue.

Methods

Microorganism and transformation

The taxonomic characteristics of *Pseudomonas putida* KT2442 have been previously reported by Memod et al. (1986). This bacteria was used as a recipient strain for cloning into pSK2665.

The plasmid pSK2665, which harbors the 5.2-kb *Sma*I-*Eco*RI subfragment SE52, harbor all three genes needed for synthesis of poly (b-hidroxybutyric acid) (PHB) from acetyl-CoA. This plasmid was granted by Dr. Steinbüchel in *Escherichia coli* XL_1-blue.

Plasmid DNA isolation, transformation, agarose gel electrophoresis and other standard DNA techniques were described by Sambrook et al. (1989).

Production media and PHA observation

The olive oil mills wastewaters (alpechín) is a natural product, obtained from raw materials and other uncontrollable variables and the chemical composition of a particular "alpechín" is not constant and thus the information about the chemical composition is exhaustive. From a global point of view, this composition is as follows (Moreno et al. 1990): water (83.2%), organic matter (15%), and minerals (1,8%).

The olive oil mills wastewaters used in this study were obtained from "Jimena Ruiz" olive oil industry, Granada (Spain). Table 1 shows the principal characteristics of this "alpechín" previously described (Martinez-Nieto et al. 1993).

The residue used as medium were supplemented with some components to get the production medium (A-50) which contains glucose 1% (w/v), yeast extract 1% (w/v) and NH_4Cl 0.5% (w/v) in a solution of "alpechín" 50% (v/v). The strain was cultured in shake-flasks 120 r.p.m. at 30ºC. PHA granules can be observed by the Burdon´s staining method (Baker, 1967).

Table 1. Physical and chemical characteristics of the "alpechín".

pH	5.26
COD (g per litre)	212.25
BOD (g per litre)	179.45
Total solids (g per litre)	55.59
Volatile solids (g per litre)	44.46
Non-volatile solids (g per litre)	11.13
Settled solids (g per litre)	7.75
Suspended solids (g per litre)	2.44
Dissolved solids (g per litre)	45.40
Total phenolic compounds	0.7%

PHA recovery

Cells were collected by centrifugation and liofilization. This biomass was determinated by gravimetry. 0.2g of biomass was suspended in 5 mL of 0.2% (w/v) sodium hypochlorite. After 1 h at 37ºC, to allow the total lysis of the suspension, PHA granules were collected by centrifugation (2000 x g). The pellet was washed with distilled water, acetone and ethanol, and the final pellet was dissolved in chloroform, with the unsoluble remains being discarded. The chloroform was evaporated at room temperature and PHA weight was obtained by gravimetry (García Lillo and Rodriguez Valera, 1990).

Results and Discussion

Pseudomonas putida KT2442 was transformed with pSK2665. The recombinant strain had the ability to grow at media with a high concentration of alpechín with PHA granules formation. The results are shown in the Table 2 .

The potential for the production of new PHA seems to be limited by the availability and costs of chemicals which can be provided as precursor substrates to the bacteria, rather than by the substrate range of PHA synthases. Therefore, the chances of obtaining PHA with new HA or unusual combination of HA in the future, will depend on the successful screening for bacteria which synthesize these precursor substrates endogenously from simple and cheap carbon sources. Complex carbon sources, which occurs in some environments, can provided as precursor substrates (Steinbüchel and Valentin, 1995).

Table 2. PHA production of *Pseudomonas putida* KT2442 and the transformed strain (pSK2665) in medium A-50.

Strain	Culture time (hours)	Biomass (g/L)	PHA content (% dry wt)	PHA yield (mg/L)
KT[1]	48	1.7742	1.52	26.97
KT[1]	72	1.3282	1.60	21.25
KTT[2]	48	0.2121	0.88	1.87
KTT[2]	72	4.2495	3.59	126.95

[1]*Pseudomonas putida* KT2442
[2]*Pseudomonas putida* KT2442 harboring the plasmid pSK2665

"Alpechín" is a cheap and complex carbon source and *Pseudomonas putida* KT2442 harboring the plasmid pSK2665 is found to grow at high concentration of this residue and accumulate PHA granules. This residue is toxic to most microorganisms but *P. putida* can grow well on such a complex material.

We can observe that *Pseudomonas putida* KT2442 harboring the plasmid pSK2665 increase the PHA production against the parental strain after 72 hours. The transformed strain was initially afected by growing at wastewater "alpechín" but, with more than 48 hours, extracromosomic genes for PHA accumulation, allow us to obtain a better production.

This is the first reference to a *Pseudomonas* able to grow in a residue as "alpechín" and also producing PHA polymer. Therefore, "alpechín" could most probably serve as an inexpensive substrate for polymers increasing then the lykelyhood of producing biopolastics at competitive prices.

In a future the production could be improved by the continuous culture of *P. putida* in "alpechín" to get results for the industrial yield. Using this inexpensive waste material, a significant cost reduction could be obtained. The characteristics of the PHA produced are another question we are searching.

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