Expression of exogenously introduced β-galactosidase gene in a tropical, marine, oil-degrading strain of *Yarrowia lipolytica* NCIM 3589

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We have transformed an oil-degrading, tropical, marine strain of *Yarrowia lipolytica* NCIM 3589, isolated from the oil drilling platform of the Bombay High region. A β-galactosidase gene with a promoter from the yeast *Saccharomyces cerevisiae* has been expressed in this organism. This opens up possibilities of developing easy selection criteria for releasing this organism in the natural environments for bioremediation and oil prospecting. Since the gene is expressed in the yeast and the mycelial forms, it would be very useful for industrial fermentations using oil waste as a substrate.

ENVIRONMENTAL pollution is a cause of major concern affecting ecosystems globally. Oil spill, an offshoot of this environmental pollution caused by spillage from tankers, release of effluents and offshore drilling activities is adversely affecting the aquatic ecosystems[1]. Chemical or physical means of controlling oil spills are rapidly being developed but they have the disadvantage that these chemicals leave toxic wastes which may not be biodegradable. Physical means are only useful in clearing up small areas. Bioremediation, i.e. the use of micro-organisms to clear oil spills[2] is widely gaining acceptance. Several bacteria, fungi and yeasts are found in abundance in the marine environment and have the capability of degrading oil. The concentration of yeasts is particularly high in areas containing organic matter. These yeasts utilize hydrocarbons as a sole carbon source, thereby degrading them. Many genera like *Pichia*, *Debaromyces*, *Candida* and *Sporobolomyces* have been found to degrade hydrocarbons[3]. *Yarrowia lipolytica* strain NCIM 3589 is one such tropical, marine strain isolated from the Bombay High region and found to degrade up to 82% of the aliphatic fraction of the crude oil. It therefore has great potential for bioremediation[3]. It is a dimorphic fungus which grows in the yeast or the mycelial forms. It also differs from the type strain in having a double-stranded RNA plasmid[3]. Given the potential biotechnological applications of this strain, genetic manipulation would prove to be useful for bioremediation and oil prospecting.

The genetic manipulation of any organism is based on stable introduction of foreign DNA into the cell through transformation[4–6]. We describe here the

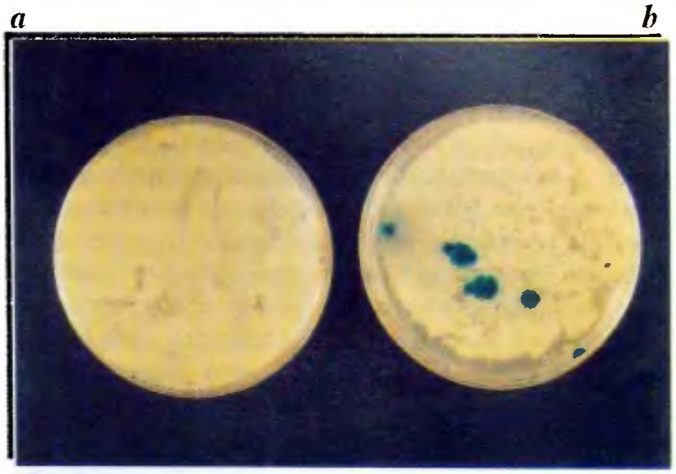

Figure 1. Transformation of *Y. lipolytica*. Cells were treated with 0.25 M lithium acetate for 1 h, the DNA added along with 20% PEG for 30 min after which the cells were heat shocked for 15 min at 42°C. Cells were washed and plated on GYP + X-gal and incubated at room temperature for 5 days. *a*, Control cells, *b*, Transformants expressing β-galactosidase.

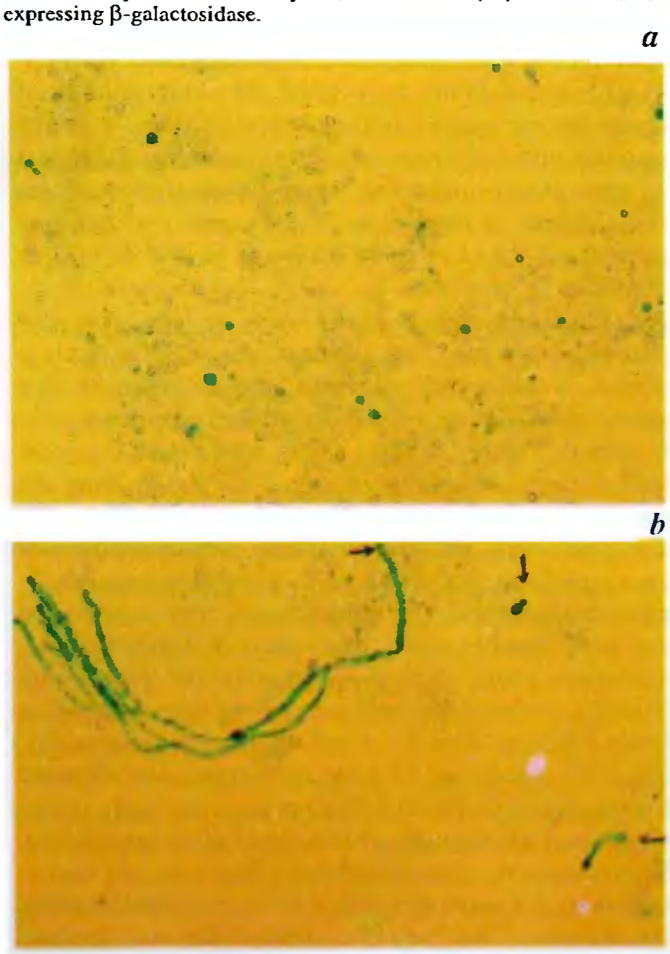

Figure 2. Staining of cells for β-galactosidase. Cells were fixed in 3.7% formaldehyde for 10 min on ice, washed, treated with the substrate (50 mM K_3FeCN_6, 50 mM K_4FeCN_6, 5 mM $MgCl_2$, 1.6% X-gal in McIvans buffer) for β-galactosidase at 37°C for 14–16 h, washed again with PBS, and observed under the Zeiss microscope, 40X. *a*, Yeast form of *Y. lipolytica* showing staining for β-galactosidase; *b*, The mycelial form of *Y. lipolytica* showing β-galactosidase staining: 1, the budding form, 2, the intermediate stage in the conversion from yeast to mycelial form and 3, the mycelial form.

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development of a stable transformation system and expression of β-galactosidase in the yeast as well as the mycelial forms of *Yarrowia lipolytica*. This will make the development of vectors for this natural, marine isolate of *Y. lipolytica* easier. The mycelial form will also be amenable for fermentations, making it possible to get industrially-important chemicals using oil waste as a substrate. *Yarrowia* cells were transformed by employing a protocol used originally for *Saccharomyces cerevisiae* with a few modifications[7]. Liquid and solid (1.2% agar) media (1% glucose, 0.3% yeast extract, 0.5% peptone) have been employed for growth. 1.6% X-gal was used as an indicator on solid media wherever required. A 10.4 kb plasmid from *S. cerevisiae* with a 2 μ plasmid origin of replication and β-galactosidase gene[8] was initially introduced in *E. coli* by transformation, the plasmid reextracted from *E. coli* used to transform *Yarrowia* cells. Distinct blue colonies of transformants were observed on these plates (Figure 1). Since *Y. lipolytica* does not carry an endogenous β-galactosidase, the non-transformant colonies were white. The blue colonies of transformants were picked up, subcultured and found to maintain blue colour on repeated subculturing for atleast 30 generations, indicating that β-galactosidase expression is stable in these cells. The frequency of transformation was found to be in the range of 10^{-5} to 10^{-6} in different experiments. To check for presence of β-galactosidase enzyme activity intracellularly, the cells were stained with a substrate for β-galactosidase (Figure 2) and observed under the microscope. The yeast form is seen in Figure 2 *a*, while the mycelial form is seen in Figure 2 *b*. This figure also shows the yeast form (1), the intermediate stage of conversion from the yeast to the mycelial form (2) and the mycelial form (3). The single cells and filaments show a distinct blue colour, indicating that these cells are expressing the β-galactosidase gene. The transformants were subcultured and were found to retain the property of β-galactosidase expression stably over many generations. The results obtained in this study indicate that we have achieved a stable transformation for a natural, tropical, marine isolate of *Y. lipolytica*. This strain degrades the aliphatic fraction from the Bombay High region efficiently (up to 82%). Such oil-degrading strains have great potential for bioremediation. Bioremediation – the use of micro-organisms for controlling oil spills is ecofriendly. Since the organisms are already present in the environment, there is no toxic waste resulting from the biodegradation and no tedious physical process involved. There are reports on genetic manipulation on the type strain of *Y. lipolytica*[9–15]. We have been able to genetically manipulate a natural marine strain isolated from the Bombay High oil platform. Having a stable transformation system in a natural isolate from the marine environments is important for genetic manipulation as other reporter genes can be introduced in this organism, and it also provides a tool for strain improvement. Introduction of β-galactosidase reporter gene is useful as it can be used as an indicator for releasing this organism in the environment for degradation of oil spills. Since this strain expresses the foreign gene in the yeast as well as the mycelial forms, it could prove very useful for industrial fermentations using oil-containing waste as a substrate.

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