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FUSION OF TWO YEAST STRAINS FOR ENHANCED CRUDE OIL DEGRADATION

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ABSTRACT

A hybrid strain S_{24} was developed from *Saccharomyces cerevisiae* Y_{5A} and *Saccharomyces carlsbergensis* Y_{7B}, using protoplast fusion technique. The yeast protoplasts were prepared using gut juice from *Helix pomatia* (Land Snail) as cell digesting enzyme. The ability of the wild and the hybrid strains to degrade medium and light crude oil was determined gravimetrically. The parental strains Y_{5A} and Y_{7A} caused 56.9 and 52.6% weight loss respectively of light crude, while the hybrid caused 72.4% weight loss which was 19.3% and 16.6% higher than Y_{5A} and Y_{7B} respectively. Strains Y_{5A} and Y_{7B} also caused 46.7 and 4.1% weight loss respectively of medium crude while S24 caused 63.3% weight loss, which was 19.2 and 15.3% higher than the Y_{5A} and Y_{7B}. The results indicate that hybrid strains are potential candidates for crude oil pollution abatement programmes in oil spilled sites.

KEYWORDS: Protoplast fusion, *Saccharomyces*, hybrid strains, Snail juice, Biodegradation.

INTRODUCTION

The demand for crude oil as a source of revenue, energy and principal raw material for petrochemical, industries has brought about increased production levels in recent times, resulting in the pollution of the environment. The incidence of accidental oil spillages and subsequent contamination of the biota has become very rampant in Nigeria, especially in the Niger Delta region where massive oil exploration, exploitation and production are carried out. Considerable efforts have been devoted to curbing the adverse effects of oil spill and its subsequent removal from the environment, yet the effects are still felt in the environment especially the effects due to the residual crude oil (Odu, 1972). The current emphasis in integrated oil pollution abatement is on microbial seeding of polluted sites. However, the challenge to this process is finding the right "candidate" for the job. This is why the development and evaluation of yeast strains for enhanced crude oil degradation is important.

With the advancement in Microbiology and Biotechnology, various genetic manipulation techniques have been employed in developing novel microorganism with improved biodegradation capabilities. The use of protoplast fusion techniques to enhance the degradation of ligninocellulose by Streptomyces species has been reported by Crawfod (1984) while Ochi (1982), Keller *et al.*, (1983) respectively, reported on the use of fusion induced Streptomyces species to produce modified antibiotics. Studies have also been reported on the isolation and identification of yeasts capable of degrading crude oil (Cook *et al.*, 1973, Amanchukwu *et al.*, 1988). Davies and Westlake (1979) successfully utilized *Aspergillus* species to degrade crude oil. Komagata *et al.* (1964) used the assimilation of hydrocarbon by yeasts as a preliminary screening method for different species of yeats while Miller and Johnson (1966) reported on the utilization of normal alkanes as sole carbon source by yeasts. Similar reports have been made by Itah (1999), Itah and Essien (2001) and Essien *et al* (2003).

From the above studies, it is evident that indigenous yeasts strains are capable of degrading a variety of hydrocarbons and thus play significant role in the control of environmental pollution. However the rate of such biodegradation is often very slow and does not allow for prompt treatment and complete removal of the enormous oily wastes and residual hydrocarbons from our environment. While there are many reports in the literature about the utilization of petroleum hydrocarbon by bacteria and few indigenous yeast strains, there seems to be paucity of information on the ability of fusion – induced recombinant yeasts strains to degrade crude oil. The objective of this study therefore, is to use protoplast fusion technique to develop a hybrid yeasts strain with enhanced crude oil degrading potential.

This work hopes to address the problem of incomplete biodegradation of crude oil in the environment by developing a superbug yeast strain that would systematically and completely degrade individual components of the crude oil, leaving only non-toxic products in the environment.

MATERIALS AND METHODS

Sample Collection

Crude Oil

Medium crude oil (Bony medium crude) was obtained from the Nigeria National Petroleum Corporation (NNPC) Research and Development Unit, Port Harcourt, whilst the light crude oil (Qua Iboe light Crude) was obtained from the Mobil Producing Nigeria Unlimited, Qua Iboe Terminal, Eket. Samples of the two crude oil types were collected in sterile sampling bottles and transported in ice-packed containers to the laboratory, within four hours of collection. The samples

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were stored at refrigeration temperature (4°C) until the commencement of the test.

Microorganisms

Saccharomyces cerevisiae Y_{5A} was isolated from Nigeria fresh palmwine, whilst *Saccharomyces carlsbergensis* Y_{7B} was supplied by the production department, Champion Breweries Plc, Uyo, Akwa Ibom State. The identities of these organisms were confirmed using their colonial morphology, microscopic examination, biochemical characteristics and identification scheme provided by Kreger – Van (1984) for yeast taxonomy.

Media

The media used in this tudy were the oxoid brand of malt extract agar, Sarbouraud and yeast extract peptone dextrose (YEPD) agar.

Enzymes

The cell wall digesting enzymes used in the work was the gut juice of *Helix pomatia* (Eddy and Williamson, 1957), obtained by crude extraction while clarex, a pectic enzyme supplied by Champion Breweries Plc, Uyo, was used to deflocculate the yeast cells.

Sterilization of enzymes, crude oil and media

The enzymes were sterilized by membrane filtration and the filtrate preserved at 4°C. All the media used in this work and the crude oil were sterilized by autoclaving at 121°C for 15 minutes under 15p.s.i. pressure.

Determination of enzyme concentration in the juice

This was done by reading the absorbance of the dilute gut juice at 540nm using spectrophotometer (Alpha series CE 2343) and converting the optical density reading to enzyme concentration using the standard protein curve described by Lowry's method (AOAC, 1984).

Determination of cellulase activity of the snail juice

The cellulase activity was determined according to the method of Herr *et al.* (1978) by measuring the amount of reducing sugar librated from the carboxymethyl cellulose (CMC).

Isolation and preservation of yeast protoplast

The yeast protoplasts were isolated using the method of Eddy and Williamson (1957) with some modifications. The modifications made were the incorporation of 3.0ml of 0.5M rhamnose, 1.0ml clarex and 1.0ml of 0.6mg/ml snail enzyme into the YEPD medium in order to prepare the cells for protoplasting. In this method, 1.0ml each of a 24 hours – old cultures of Y_{5A} and Y_{7B} were inoculated separately into 500ml of YEPD medium containing 3.0ml rhamnose, 1.0ml of clarex and 1.0ml of 0.6mg/ml of the snail enzyme. The mixture was incubated at 25°C with gentle shaking at 300rpm on orbital shaker (S01, Stuart Scientific, England) for 36 hours. Cell count was determined at 4 hourly interval using clinical Haemocytometer. When a population density of 6.5×10^6 cells/ml was attained the cells were harvested, washed three times with 10.0ml of 10 millimolar EDTA solution by centrifugation at 1000 x g for 5 minutes to deflocculate the yeast cells. The initial washing was followed by a final wash (once) with sterile distilled water. thereafter the supernatant was decanted and the cells dried in hot air oven maintained at 40°C. ten milligram each of the washed cells was re-suspended in 5.0ml protoplasting buffer (sorbitol-phosphate buffer, pH 7.4) and kept for 10 hours.

To 5.0ml of the buffered yeast suspension, 10.0ml of protplasting forming solution consisting of 10.0ml protoplasting buffer, 5.0ml sterile water, 0.2mg/ml of snail enzyme and 0.2ml of one molar 2-mercaptoethanol was added. The mixture was incubated for 48 hours at room temperature with gentle shaking on orbital shaker at 75rpm. Control tests were similarly treated but without snail juice. After incubation the cells were examined microscopically to confirm their conversion to protoplast.

To preserve the protoplasts formed, the protoplast suspensions were centrifuged at 1000 x g of for minutes and washed 5 times with 10.0ml of protoplasting buffer. The final pellets were taken up separately in 10.0ml of this buffer and preserved under refrigeration temperature.

Fusion of yeast protoplasts

In this test, 0.5ml each of the protoplast suspensions from Y_{7B} were mixed and centrifuged at 1000 x g for 5 minutes. The supernatant fluid was carefully pipetted out with a sterile pipette while the

Table 1: Comparison of the Contents of Parental and Regenerated Cell Walls

CONTENT	NORMAL CELL WALLS		REGENERATED CELL WALL
	Y_{5A}	Y_{7B}	
Mannose	+	+	+
Glucose	++	++	++
Nitrogen (%Dry wt.)	1.25[a]	1.42	2.50
Phosphorus (%Dry wt.)	0.15	0.18	0.03
Amino acid residue	+++	+++	±
Lipid	++	++	+++

[a]Values refer to three independent preparations

+++: High

++: Moderate

+: Minimal

±: Relatively negligible

resulting pellets were carefully suspended in 10ml of fusion solution consisting of 35% ($^w/_v$) polyethylene glycol (PEG) 6000, 3% ($^w/_v$) pure ethylene glycol and 5.0ml of $CaCl_2$ dissolved in 10milli molar tris-HCl (pH 7.5) and incubated at 30°C for 3 days without shaking. The fusion mixture was examined periodically, after every six-hours, under a well illuminated microscope to confirm fusion.

Regeneration of cell on the fused protoplasts

For the regeneration of cell wall, 0.1ml of the protoplast suspension aggregates was mixed with 10.0ml of molten hypertonic cell wall regeneration agar medium consisting of 1% ($^w/_v$) yeast extract, 2% mycological peptone, 2% glucose, 3% agar-agar, 1% sorbital and 1 litre of distilled water. Thereafter the mixture was immediately overlaid onto petridishes containing pre-solidified media of the same composition. The plates were allowed to set, thenincubated in an inverted position at 25°C for 7 days.

Examination of fusion recombinant

The regenerated colonies were isolated from the surface of the hypertonic regeneration medium and subcultured into a variety of selective media supplemented with 2% glucose, and incubated without shaking, at 25°C for 3 days. After incubation, the cultures were subjected to microscopic examination and biochemical analysis.

Growth of parent and recombinant strains in crude oil

About 1.4 x 10^6 cells of the active inoculum of the wild and the recombinant strains were inoculated separately into 25.0ml mineral salt medium containing 1.0% sterile medium and light crude oil samples, and incubated at 25°C for 16 days in a shaker incubator set at 100rpm. After this period the residual crude oil was extracted with carbon tetrachloride (CCl_4) and the optical density readings were converted to weight lose by reading from a standard crude oil weight loss curve.

Evaluation of crude oil degradation by the wild and recombinant yeast strains

In this test, three sets of tubes containing 16 tubes each were set up. Each set was inoculated with 0.1ml of a 24 hour old broth culture of the parent and the recombinant strains. Uninoculated tubes served as control. The tubes were incubated at room temperature for 16 days on orbital shaker maintained at 100rpm. Two tubes from each set were analyzed gravimetrically every 4 days for residual crude oil.

The percentage weight loss was calculated as weight of crude oil (control) minus weighed of crude oil (degraded) divided by weight of crude oil (control) multiplied by 100.

The effect of the medium and light crude oil on the growth of the three yeast strains was determined according to the method of Okpokwasili and Okorie (1988) by carrying out total viable count of the test organisms in each of the test tubes after incubation. The growth profile was obtained by plotting the number of viable yeast cells against various concentrations of the crude oil types.

Statistical analysis

The student t-test statistic at 1.0% level, as described by Phillips (1973), was employed to determine the significant difference between the biodegradation of light and medium crude oil by the three yeast strains.

RESULTS

Isolation and examination of protoplasts

After 24 hours incubation, the protoplasts were seen under the microscope, as spherical densely cytoplasmic structures without any overlaying wall materials. Further examination of samples withdrawn at intervals of 4 hours from cultures held at 25°C for up to 7 days showed morphological alterations on the protoplasts. The surfaces of the protoplasts displayed irregular swellings which later developed into a mass of irregularly shaped outgrowths, the protoplast themselves increasing in volume.

Fusion of the protoplast

Periodic examination of samples withdraw at intervals of 6 hours showed that the sequence of fusion events started with agglutination of the protoplasts and the formation of aggregates of various extents. Complete fusion was obtained after 48 hours at 35% ($^w/_v$) PEG 6000 in the presence of 10 milli-molar $CaCl_2$ and 3% ($^w/_v$) pure ethylene glycol.

Cell wall regeneration

Hypertonic YEPD agar gave a better wall regeneration than the malt extract agar. The result of the comparative studies on the contents of the wild and regenerated cell walls is presented in Table 1.

Selection of recombinant strain

As an aid to the selection of the resulting recombinants, both Y_{5A} and Y_{7B} were unable to grow on a medium containing 1% glycerol, a non-fermentable carbon source. The recombinant strain S_{24} showed differences when compared with the parents.

Biodegradation of crude oil type by the test organisms

Results of the biodegradation of light and medium crude oil samples by Y_{5A}, Y_{7B} and S_{24} are presented in Figures 1 and 2. All the test organisms in this work degraded medium and light crude oil to varying levels. Figure 1 also illustrates the rate of utilization of medium and light crude by the three yeast strain after 16 days incubation. The results revealed that both the parents and the recombinant strain exhibited preference for the light crude over the medium crude. The biodegradation of the two types of crude oil by S_{24} was more rapid and extensive than that by Y_{5A} and Y_{7B} respectively.

The results of weight loss from light and medium crude oil due to the growth of the three yeast strains is also presented in Figure 2. The result revealed that the weight loss caused by S_{24} was comparatively higher than that caused by Y_{5A} and Y_{7B}. While Y_{5A} and Y_{7B} caused a weight loss of 56.9% and 52.6% respectively, of light crude the percentage weight loss of the light crude caused by S_{24} was 72.4%. average weight loss of

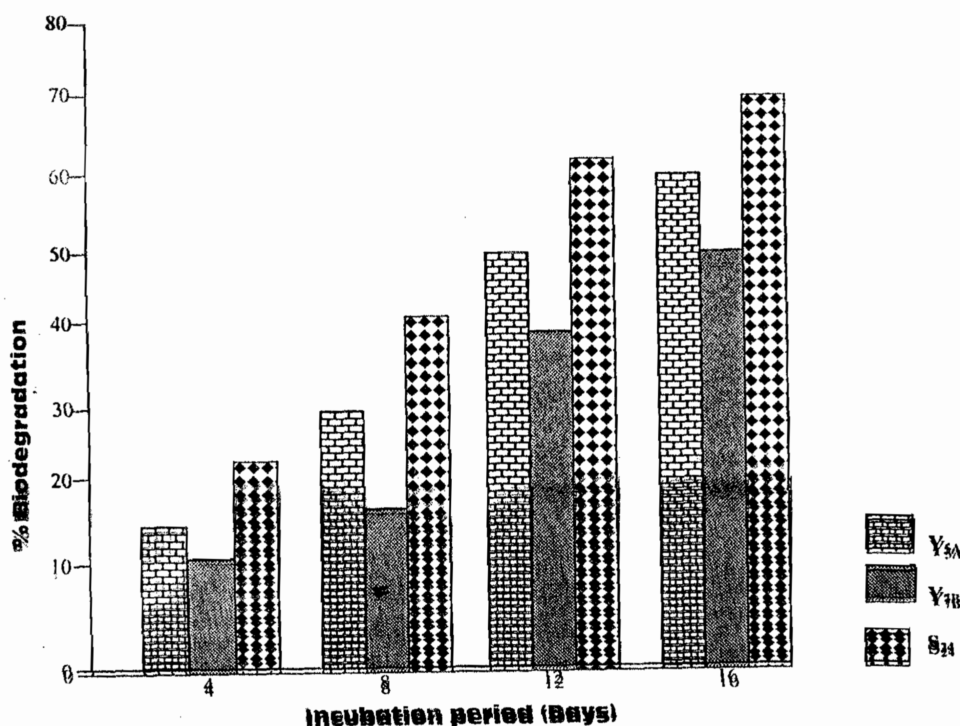

Figure 1: *Biodegradation of Light Crude Oil by Y_{5A}, Y_{7B} and S_{24}*

medium crude oil resulting from 16 days growth by S_{24} was 63.3% compared to 46.7% and 44.1% caused by the parental strains Y_{5A} and Y_{7B} respectively.

The result of the statistical analysis carried out in this work showed that the percentage biodegradation of medium and light crude oil by S_{24} significantly differed ($P<0.01$) from the percentage biodegradation exhibited by Y_{6A} and Y_{7B}. There was no significant difference ($P>0.01$) in the percentage biodegradation of light and medium crude oil by Y_{6A} and Y_{7B}.

DISCUSSION

In this study, the ability of the wild yeast Y_{6A} and Y_{7B} and the fusion induced recombinant strain S_{24} to degrade medium and light crude oil has been demonstrated. The import of this work is that PEG 6000-induced protoplast fusion technique can be used to develop novel yeast strain with enhanced crude oil degrading capability. The highest biodegradation potential recorded by S_{24} suggests that the recombinant possessed most efficient enzymes for biodegradation. Protoplast fusion might have modified the genes coding for the synthesis or regulation of one or more enzymes involved in the biodegradation of crude oil. This is obvious because each time the recombinant was tested for crude oil degradation it showed higher potential to cause more weight loss at 2-5% concentrations of light and medium crude. A similar observation was made by Ijah and Antai (1990), who used Streptomyces mutant to enhance crude oil degradation. However, higher concentrations up to 10% was inhibitory to the recombinant and the wild strains. This might be due to the fact that at high substrate concentration, microbial growth becomes inhibited probably because of enzyme inactivation (Prescott *et al.*, 1999).

Atlas & Bartha (1972), also reported that at high concentration, crude oil becomes extremely insoluble in aqueous medium, resulting in non-emulsification which may obstruct enzymatic degradation. The varying degrees to which each crude oil type in this study was expected because microorganisms have varying preference for different carbon substrates for metabolism and energy production Ijah and Okang (1993).

The relatively low percentage of total lipid in S_{24} when compared with parental strains seems to play some role in the crude oil uptake. The low lipid content probably increased the ability of the recombinant to utilize more crude oil which increased the rate of biodegradation.

The result obtained from this study also reveals that the gut-juice of *Helix pomatia* can digest the cell wall of *S. cerevisiae* and *S. carlsbergensis*, leaving the protoplasts intact, especially when young growing cells are used. The ability of the gut juice of land snail to digest the cell wall may be due to the high cellulase activity of the juice.

SUMMARY AND CONCLUSION

The response of fusion induced recombinant strain S_{24} to the biodegradation of crude oil types from two completely different geographical locations revealed that successful fusion and cell wall regeneration had occurred, and that protoplast fusion technique could be used to develop novel yeast strains with improved crude

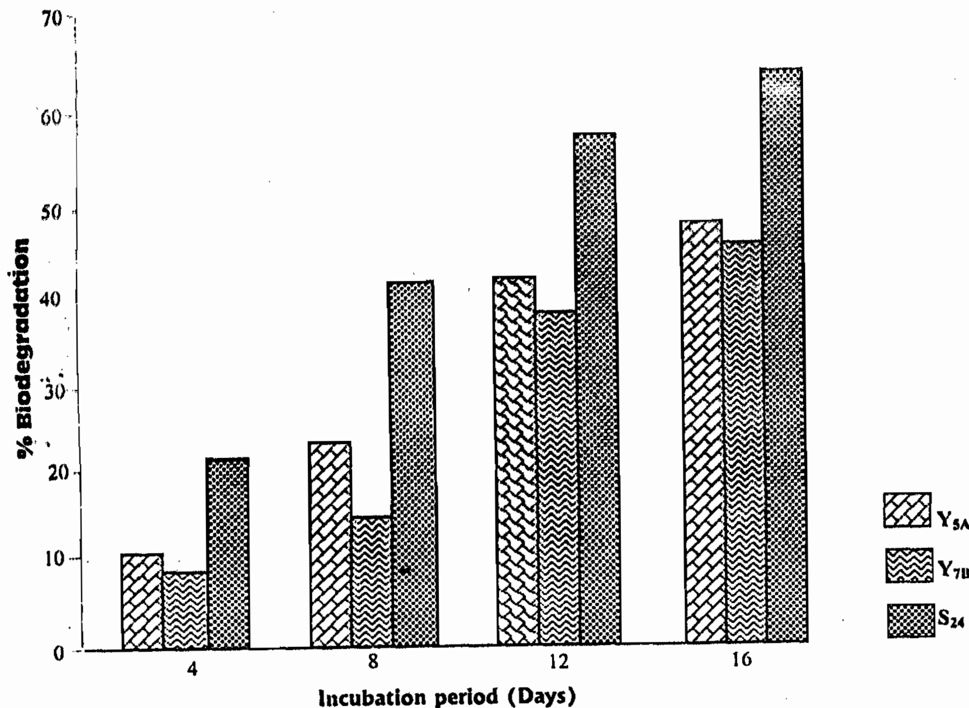

Figure 2: *Biodegradation of Medium Crude Oil by Y_{5A}, Y_{7B} and S_{24}.*

oil utilization potential. Hence such strains could be improved upon and applied in bioremediation processes aimed at the removal of residual crude oil in spill sites. However, further work should be carried and temperature on the biodegredation potential of the recombinant strain.

Since this study has shown that the fusion induced recombinant yeast strain S_{24} can exhibit high growth rate on medium and light crudes, and appreciably degrade these crude oil types, the organism is therefore recommended for further studies on the safety of using for environment pollution abatement.

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