Swedish University of Agricultural Sciences
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Abstract

Proper nutrition plays a key role in the growth of fish. However, limited supply of raw material for fish feed has posed problem for the ever-growing Aquaculture. Waste water from food industries (e.g. diaries and slaughterhouses) contains considerable amounts of proteins and fats to be used as fish feed. Similarly, glycerol which is a by-product in biodiesel production can be used as carbon source by yeast. Linseed oil can be useful as a source of fatty acids.

In this study we investigate the potential of waste from paper and beer industries as a growth medium for yeast. We used two different concentrations of Glycerol and Linseed oil. Media was inoculated with yeast and cultured for 48 hours. To determine the growth of yeast, OD (Optical Density) was measured for each sample at three hours intervals.

Our results show that waste from beer industry is very promising as compared to waste from paper industry for the growth of yeast. Moreover, all strains of yeast showed better growth when lower concentrations of glycerol and linseed oil were added to beer water as compared to high concentrations.

Keywords: Fish Feed, Glycerol, Linseed Oil, Beer Water, Waste from paper industry, Yeast

CONTENTS

Introduction

Fish Production

Although aquaculture in world is increasing constantly, it is still facing problems due to limited supply of raw material for fish feed (Tacon and Metian, 2008). For promoting normal growth of fish, proper nutrition plays a key role. In certain fish production, Brewer's yeast is used as a fish feed (Nayar et al. 1998). Food industries (e.g. dairies and slaughterhouse) release waste-water, which contains considerable amounts of proteins and fats. Conversion of fat into yeast biomass could be beneficial, because yeast biomass can be use as fish feed. Different carbon sources including glycerol have been used for the highest growth yield of *C. trophicals* (Rydin et al. 1990). Brewer's yeast has different immunostimulating compounds e.g. nucleic acid, b-glucans and mannan oligosaccharides (White et al. 2002). These compounds may enhance growth of different fish species and therefore can serve as best health promoters for fish culture (Lara-Flores et al. 2002). For mass production of nematode *Panagrellus redivivus*, brewer's yeast is suitable as food source. This nematode is used as a source of food for feeding farm fish (Ricci et al. 2003).

Glycerol as by product of Biodiesel Production and its Utilization

Biodiesel is derived from animal fats or vegetable oils, and is a domestic renewable fuel for diesel engine which meets ASTM (American Society for Testing and Materials) specifications (National Biodiesel Board USA). In recent years, Biodiesel attracted increasing attention as an alternative fuel. For instance, in United States, annual production of Biodiesel increased from 75 million gallons in 2005 to 700 million gallons in 2008 (National Biodiesel Board USA). Major by-product in biodiesel production is crude glycerol, which is 10% wt of vegetable oil. 1 kg of crude glycerol is formed as by-product for each 9 kg biodiesel produced (Dasari et al. 2005). During recent years, annual consumption of glycerol by world remained constant which is 600,000 tons per year. It can be use in different industries e.g. production of soaps, cosmetics and pharmaceuticals etc (Bondioli 2003). Through thermo chemicals methods, crude glycerol can be convert into value added products (Johnson and Taconi 2007). Glycerol can be utilized as a carbon and energy source by number of yeasts e.g. *Candida utilis* and *Saccharomyces cerevisiae* (Gancedo et al. 1968).

Linseed Oil

Linseed oil is clear yellowish oil. It comes from dried ripe seeds of flax plant (*Linum usitatissimum*, *Linaceae).* Both omega-3 and omega-6 fatty acids are present in Linseed oil (University of Maryland Medical Centre). Nearly 60% α-Linolenic acid (ALA) is present in Linseed Oil. ALA is an unsaturated fatty acid. It is believed that linolenic acid is responsible for increase eicosapentaenoic (EPA) production (Shimizu et al. 1989b).

It is believed that humans and animals need dietary unsaturated fatty acids for body maintenance and normal growth. Information shows that unsaturated fatty acids play a vital role in alleviation of autoimmune disease, prevention of renal and cardiovascular diseases (Bang et al. 1976, Stoof et al. 1989, Prickett et al. 1981, Priekett et al. 1983).

Saccharomyces cerevisiae can only synthesize mono unsaturated fatty acids (Resnick and Mottimer 1966, Choi et al. 1996, Fujimori et al. 1997). On other hand, polyunsaturated fatty acids e.g. linolenic acid and linoeic acid can be synthesize by yeast i.e. *Hansenula polymorpha* and *Candida (Yarrowia) lipolytica* (Wijeyaratne et al. 1986, Kates and Paradis 1973, Pugh and Kates 1973).

A previous study showed that yeast grown on medium having fish oil as a supplement has high content of lipids, essential fatty acids and unsaturated fatty acids. Fish use this yeast as their feed (Imada et al. 1979).

Aim of project

To study growth of three yeast strains on substrates from paper and beer industries using two concentrations of glycerol and Linseed Oil as carbon source in order to produce yeast biomass for fish feed.

Materials and Methods

Strain used

Three Yeast strains were used in this experiment. These strains were YMO2, YMO3 and YMO4, which were provided by Department of Microbiology SLU Uppsala. All the strains were stored in cold room at 2 °C at Department of Microbiology SLU Uppsala.

Medium used

YPD medium used in this experiment contained 10 g/l yeast extract (Oxoid Ltd England), 20 g/ l peptone (Oxoid Ltd England) and 20 g/l glucose (VWR international BVBA Belgium).

Beer Water was provided by Slottskällan Uppsala Sweden. This is by product in beer industry. It was filtered to remove large fiber particles and then stored in cold room at 2 °C.

Waste from paper industry (referred to as TMP in this thesis) was provided by Hallstaviks Pappersbrukt in Hallstavik. It was filtered to remove large fiber particles and then stored in cold room at 2 °C.

Media without Glycerol and Linseed Oil were used as a control.

Growth media which were used for growth experiments are shown in Table1.

Media Code	Medium	Addition	Amount (g/l)
1	YPD	-	-
1a	YPD	Glycerol	30
1b	YPD	Glycerol	0.3
1c	YPD	Linseed Oil	30
1d	YPD	Linseed Oil	0.3
2	Beer Water (BW)	-	-
2a	BW	Glycerol	30
2b	BW	Glycerol	0.3
2c	BW	Linseed Oil	30
2d	BW	Linseed Oil	0.3
3	BW + TMP	-	-
3a	BW + TMP	Glycerol	30
3b	BW + TMP	Glycerol	0.3
3c	BW + TMP	Linseed Oil	30
3d	BW + TMP	Linseed Oil	0.3
4	TMP	-	-
4a	TMP	Glycerol	30
4b	TMP	Glycerol	0.3
4c	TMP	Linseed Oil	30
4d	TMP	Linseed Oil	0.3

Table1.Growth Media

Culture Conditions

All experiments were performed in 250 ml Erlenmeyer flasks (E-flasks). Before inoculation, Erlenmeyer flasks were autoclaved at 121 °C for 15 min. To each E-flask, 200 ml growth medium were added and inoculated with 1 ml inoculation culture. The inoculation culture was created by adding colonies from culture to 10 ml normal saline solution (9 g NaCl dissolved in 1L Distilled

water). E-flasks were incubated with shaking in an orbital shaker (148 rpm) for 48 hours. Incubation temperature for all experiments was 25 °C.

Inoculation

1ml of pre-culture was added to each growth medium. Before inoculation OD_{600} was adjusted to 0.500 for all samples. Yeast from culture plates was added to normal saline (0.9% NaCl). During O.D measurement normal saline was used as a control blank.

Freeze drying analysis

For freeze drying analysis, cells were harvested from each flask by centrifugation at 4000 g for 15 min and washed three times with 10 ml normal saline (9 g NaCl dissolved in 1L Distilled water). Freeze-dryer (Lyostar 11, FTS Kinetics USA) was used for freeze-drying. Following steps were involved in freeze-drying. 10 ml glass tubes were filled with 1 ml cell suspension. These tubes were then cooled on shelf in freeze-dryer at 5 °C per minute until temperature reached -50 °C. Tubes were then held isothermally for 50 minutes at that temperature. Primary drying was performed at -20 °C shelf temperature and 5.3 Pa pressure at chamber. Secondary drying was performed stepwise until temperature 20 °C and pressure 13 Pa. After freeze-drying completion vials were kept at 4 °C and 67 Pa. In order to estimate cell weight, glass vials were weighed before and after freeze-dying. After measuring dry weight for all samples, freeze-dried cell were used for fatty acid profile.

Results

OD measurement

All yeast strains were inoculated on 1a, 1b, 1c and 1d medium, along with 1 medium as a control. OD_{600} was measured for all samples after 48 hours as shown in fig 1.

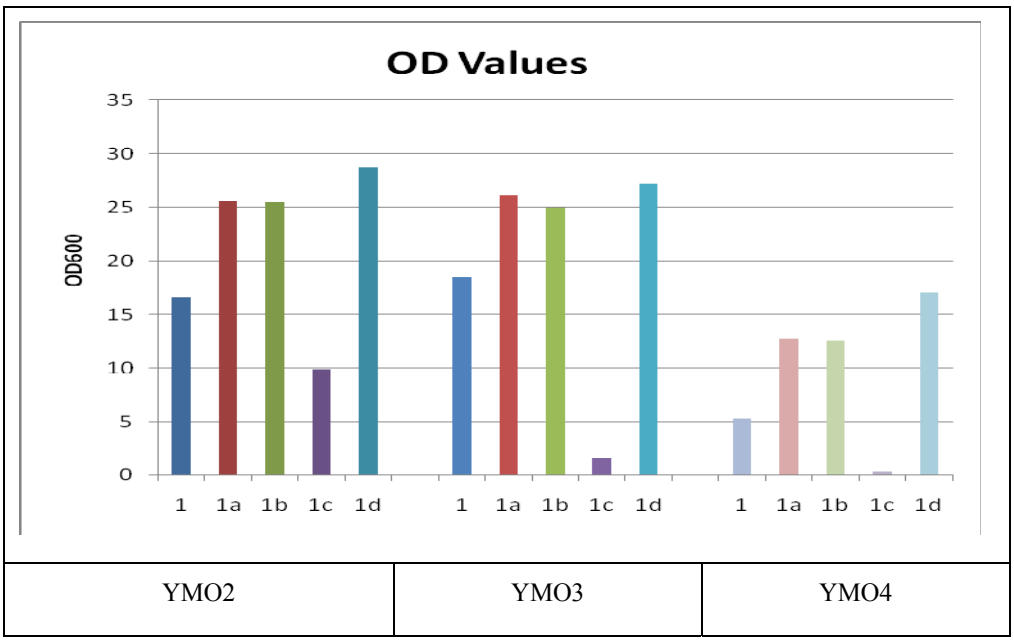

Fig1. OD_{600} values of YMO2, YMO3 and YMO4 on 1a, 1b, 1c, 1d and 1 (control) medium after 48 hours.

It was observed that after 48 h, YMO2 and YMO4 showed higher OD value on 1a, 1b and 1d medium and lower OD value on 1c media as compared to control medium. While YMO3 showed higher OD value on 1a and 1b medium and lower OD value on 1c and 1d medium as compared to control medium. By comparing all strains, YMO2 showed highest OD value on 1b, 1c and 1d medium, while YMO3 showed highest OD value on 1a medium.

In another experiment all strains were inoculated on 2a, 2b, 2c and 2d medium. In this experiment 2 media was used as a control. For all strains, OD_{600} was measured after 48 h as shown in figure 2.

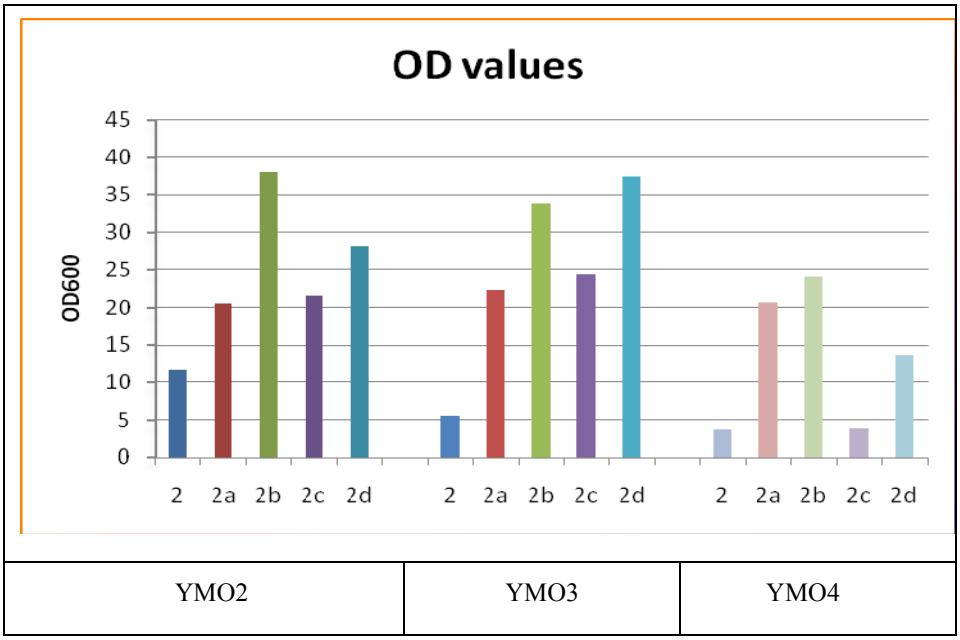

Fig2.OD_{600} values of YMO2, YMO3 and YMO4 on 2a, 2b, 2c, 2d and 2 (control) medium after 48 hours.

It was observed that after 48 h, YMO2 and YMO3 showed higher OD value on all medium as compare to control. YMO4 showed higher OD value on 2a, 2b and 2d medium as compare to control. OD value of YMO4 grown on 2c media was similar to control. By comparing all strains, YMO3 showed highest OD value on 2a, 2c and 2d medium, while YMO2 showed highest OD value on 2b media.

Next all strains were inoculated on 3a, 3b, 3c and 3d medium, along with 3 media as a control. OD_{600} was measured for all strains after 48 h as shown in figure 3.

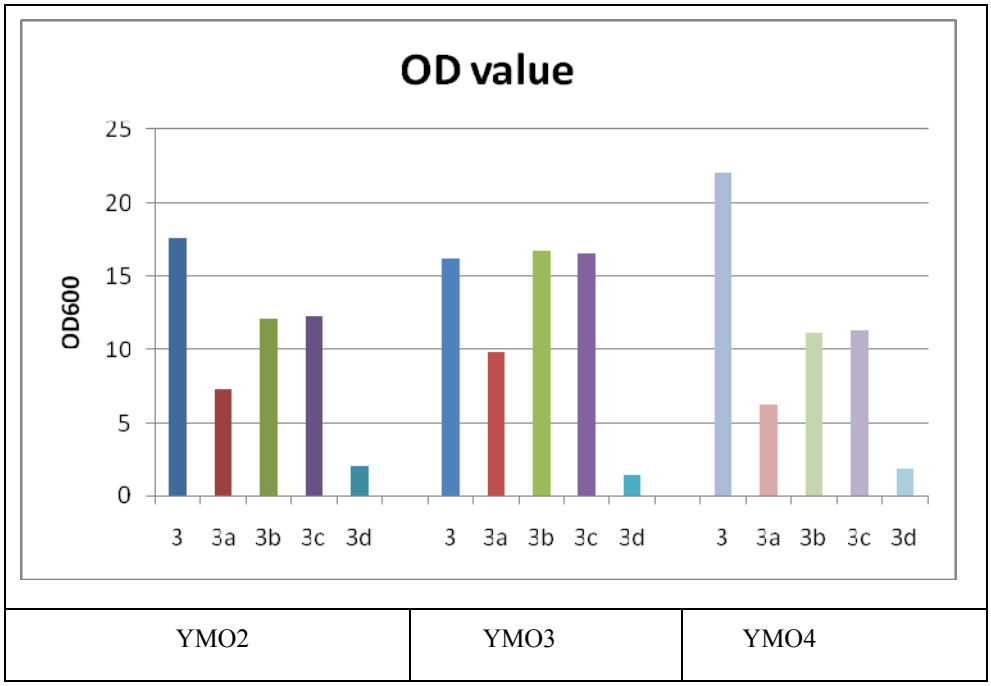

Fig3.OD_{600} values of YMO2, YMO3 and YMO4 on 3a, 3b, 3c, 3d and 3 (control) medium after 48 hours.

It was observed that after 48 h, YMO2 and YMO4 showed lower OD value on all medium as compare to control. YMO3 showed lower OD value 3a and 3c medium as compare to control. OD values of YMO3 grown on 3b and 3c medium were similar to control. By comparing all strains, YMO3 showed highest OD value on 3a, 3b and 3c medium. All strains showed similar OD value on 3d medium.

Next all strains were inoculated on 4a, 4b, 4c and 4d medium, along with 4 media as a control. All yeast strains were unable to grow on these medium. Thus no results were observed.

Cell dry Weight

Cell dry weight was measured for all yeast strains as shown in table 2.

Dry cell weight of YMO2, YMO3 and YMO4.

Strain	Medium	Total Weight (g/ml)	Standard deviation
YMO2	1	0.88	0.167571
	1a	0.71	0.0287518
	1b	0.63	0.038987
	1c	0.40	0.319541
	1d	0.76	0.032249
	2	0.39	0.209189
	2a	0.58	0.0338625
	2b	0.81	0.013663
	2c	0.32	0.283173
	2d	0.66	0.258663
	3	0.49	0.135056
	3a	0	0
	3b	0.17	0.027325
	3c	0.18	0.036148
	3d	0.013	0.005164
YMO3	1	0.75	0.202056
	1a	0.73	0.054406
	1b	0.16	0.040988
	1c	0.04	0.061968
	1d	0.59	0.181622
	2	0.25	0.073212
	2a	0.51	0.25044
	2b	0.78	0.108812

	2c	0.26	0.173051
	2d	0.89	0.044721
	3	0.46	0.036148
	3a	0.35	0.032249
	3b	0.28	0.040988
	3c	0.19	0.050859
	3d	0.0066	0.010328
YMO4	1	0.46	0.07642
	1a	0.43	0.06261
	1b	0.63	0.038987
	1c	0	0
	1d	0.49	0.020656
	2	0.19	0.005164
	2a	0.51	0.031411
	2b	0.46	0.234606
	2c	0.0033	0.005164
	2d	0.28	0
	3	0.30	0.106708
	3a	0.23	0.008944
	3b	0.23	0.013663
	3c	0.12	0.026833
	3d	0.013	0.013663

Table2. Cell dry weight of YMO2, YMO3 and YMO4 on various media (as mentioned in Table1).

By comparing all strains, it was observed that YMO3 showed highest dry cell weight on 1a, 2d, 3a, 3b and 3c medium, while YMO2 showed highest cell dry weight on 1c, 1d, 2a, 2b and 2c medium. YMO4 and YMO2 showed similar dry cell weight on 1b and 3d medium.

Discussion

In this study we attempt to identify suitable yeast strain, substrate and concentration of glycerol and Linseed Oil influencing yeast biomass. Glycerol and Linseed oil were used as a carbon and energy source. Previous studies showed that glycerol can be utilized as carbon and energy source by number of yeasts e.g. C*andida utilis*, *Saccharomyces cerevisiae, Rhodotorula glutinis, Yarrowia lipolytica* and *Sporobolomyces ruberrimus* (Gancedo et al. 1968, Easterling et al, Papanikolaou et al. 2002 and Razavi and Marc 2006).

Our results showed that all yeast strains can grow on glycerol concentration of 30 and 0.3 g/l in YPD medium (Fig 1). Previous report showed that at higher glycerol concentration i.e. 64 g/l yeast growth is repressed while at lower glycerol concentration i.e. 16 g/l yeast show best growth (Meesters et al. 1996). In this study, it was observed that by decreasing glycerol concentration from 16 g/l to 0.3 g/l yeast show's similar growth pattern.

We observed low yeast growth on BW medium with glycerol 30 g/l (Fig 2) and BW + TMP medium with glycerol 30 g/l (Fig 3). Previous study showed that cell of *Rhodotorula lactosa* became contracted when grow on 28 g/l glycerol, but cell morphology was normal at 18 and 21.5 g/l glycerol (Martelli et al. 1992). According to Grey and Sova, inhibitory effect of glycerol on *Saccharomyces cerevisiae* was by plasmolysis of cell membrane (Grey and Sova 1956).

In this study, Linseed oil was used as a carbon and energy source. According to previous report linseed oil can be utilized as carbon and energy source by fungi e.g. *Mortierella alpina and Mortierella elongata* (Bajpai et al. 1991). Previous study showed that addition of linseed oil to medium increase yeast biomass. This increase of yeast biomass was due to excess oil storage in yeast cells (Athalye et al. 2009). To confirm these results we carried out a study in which different growth media and different linseed oil concentrations were used. We observed high yeast growth on YPD (Fig 1) and BW (Fig 2) media having linseed oil concentration of 0.3g/l but when the concentration was increased to 30 g/l there was a decrease in yeast growth. When BW + TMP with linseed oil concentration of 30g/l (Fig 3) yeast showed high growth, while decreasing the concentration to 0.3g /l there was a decrease in yeast growth. From these results we hypothesized that not only linseed oil concentration affects yeast growth but different media also have an effect on the growth.

In another experiment we used by products from paper industry (TMP) as growth medium for yeast. We observed no yeast growth using this medium. Chemical analysis of this medium shows

that it contains very low amount of sugars e.g. glucose, fructose, sucrose, lactose and maltose. The concentration for all these sugars was < 0.04g / 100 g.

Conclusion

By products from paper and beer industry can be use as a substrate for yeast. Glycerol and Linseed Oil can be use as a carbon and energy source for yeast growth and increase biomass yield. Careful choice of concentration of glycerol and linseed oil is very essential otherwise yeast biomass will be significantly decreased.

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