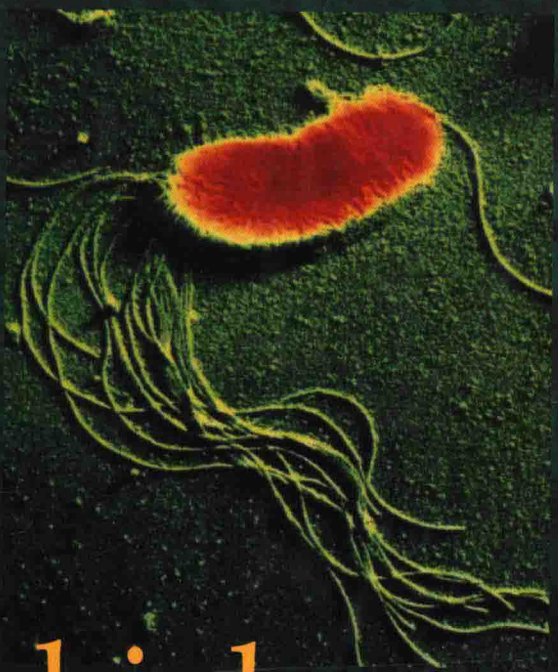

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Methods and Applications

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PREFACE

Microorganisms are a major component of our planet earth. They have wonderful metabolic capabilities that allow them to easily adapt to different environment. Activities of microorganisms are very important to almost every sector of concern to mankind. Applications of microbes in the field of science are a fascinating and are increasingly regarded as a mainstream, central tenet of biology rather than just an interesting peripheral topic. Microbes can survive extreme environmental conditions where no other organisms can survive and grow. They have the ability to synthesize unique enzymes or proteins that could enhance the biological processes and bring out biotransformation of materials in the environment. These microbes are responsible for various activities which could be either beneficial or harmful to human beings. The research into their applications is highly valued both by scientists and by academicians and industry professionals, who thrive upon the latest developments. While negative role of microbes have been known for causation diseases, beneficial role of micro-organisms have been recognized in the field of environment, agriculture, mining, medical and food industries as well. However, applied microbiology is coming to the aid of the environment through the development of bioremediation and biosorption, the use of living microorganisms to return the environment to its natural state. Microbes or their enzymes are being used to degrade toxic wastes instead of traditional processes, thus waste treatment is useful industrial asset of biotechnology. Cleaning up environmental pollutants like oil, gas and heavy metals is a considerable challenge.

Microbes are playing increasingly important roles in commercial mining operations, where they are being used in the "bioleaching" of copper, uranium, and gold ores. Certain microbes have toxic gene products, which act only on specific plants and insects. Therefore, they have an advantage over some chemicals because they can be used in limited amounts and have much more specific effects. The bacterial toxin can be concentrated for use as an insecticide spray. Microorganisms are being employed since several decades for large-scale production of a variety of biochemicals ranging from alcohols to antibiotics, enzymes and in processing of foods and feeds, etc. Microorganisms are capable of producing many common organic chemicals, like ethanol, butanol, acetic acid etc. They can also produce proteins for vaccines and other uses through a fermentation process.

From the above applications, microbes bear great potential for biotechnological applications, without microbes biotechnology would be a limited science. They not only provide the foundation for much of the basic research involved in biotechnology, but also help to create many processes which are integral to this science. Use of microbes

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to obtain a product or service of economic importance constitutes Industrial Microbiology or Microbial Biotechnology. Due to the continued research in the past few centuries, we have come to a stage to identify these organisms and the processes responsible for particular activity which could be harnessed by the industry for generation of products for well-being of the society. As researches are in progress, new findings and information are generated in laboratories which need to be collected or compiled and published for benefit of scientists, researchers and academicians. In a humble attempt papers have been invited from the scientists and academicians working in different fields of microbiology, biotechnology and related areas, compiled and presented in the present book as an edited volume. We are sure; the articles will be appreciated by the readers because of its novelty and updated information written by experts in their field.

We take pleasure to thank the authors for their cooperation and excellent contributions and for keeping to the publication schedule. The efforts of these personalities made our task much easier than it might have been. It is really a matter of privilege for us to be associated with experts in the field whose significant contributions have taken microbial biotechnology to a new height. We also thank the staff at Norosa publishing House Pvt. Ltd. and its managing Director, Mr. N.K. Mehra for their patience, flexibility, professionalism and quality concern.

H. N. Thatoi
B. B. Mishra

CONTENTS

PRODUCTION OF PYRUVIC ACID, 2-KETO ACIDS AND CHIRAL 2-HYDROXY ACIDS USING GLYCOLATE OXIDASE AND CATALASE CO-EXPRESSED IN *PICHIA PASTORIS*

*S. Das[1] and M. Subramanian[1,2]**

ABSTRACT

Glycolate oxidase (GO; (*S*)-2-Hydroxyacid oxidase, EC 1.1.3.15) is a flavin mononucleotide-dependent peroxisomal enzyme, which catalyzes the oxidation of 2-hydroxy carboxylic acids such as lactate or glycolate, to the corresponding 2-keto acids. Hydrogen peroxide formed in the above reaction is detrimental to product formation and enzyme activity unless scavenged by catalase. Hence, GO from spinach and catalase T from *Saccharomyces cerevisiae* were co-expressed in methylotrophic yeast *Pichia pastoris* strain NRRL Y-21001. Permeabilized whole cells of this recombinant *P. pastoris* were used as biocatalyst to determine the substrate specificity of 2-hydroxy acids such as 3-phenyllactic acid, 3-indolelactic acid, 2-chlorolactic acid, 2-hydroxybutyric acid and 2-hydroxydecanoic acid. GO oxidized (*S*)-enantiomers of these acids with a high degree selectivity, leaving (*R*)-isomers intact. The rate of oxidation of different substrates ranged from 1.3 to 120 %, relative to the oxidation of lactate to pyruvate. Oxidation of (*RS*)-lactic acid, (*RS*)-2-hydroxybutanoic acid, and (*RS*), & (*S*)-3-phenyllactic acid was carried out in 500 mL scale for the characterization of products and stoichiometry of the reaction. Pyruvate was produced in Kg scale as a white crystalline powder, comparable to the commercial standard. Additional purification of pyruvate was not needed; the product was recovered at high purity directly from the reaction mixture. The purity of pyruvate was confirmed by HPLC and LCMS. External addition of catalase and FMN was not required for maximum conversion of lactate to pyruvate. Given the exquisite selectivity of GO towards

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(*S*)-2-hydroxyacids, sodium borohydride was used in a one-pot reaction to create a dynamic process for production of (*R*)-2-hydroxy acids. Sodium borohydride was efficient in the reduction and recycling of 2-keto acids without compromising the enzyme activity. Preliminary studies indicated that the yields of (*R*)-2-hydroxyacids from (*RS*)-lactic acid, 2-hydroxybutanoic acid, and 3-phenyllactic acid in the dynamic one-pot process ranged from 95% to 99%.

Key words: Glycolate oxidase, *Pichia pastoris*, 2-hydroxy acid, resolution, and pyruvic acid

1. INTRODUCTION

1.1. Industrial use of pyruvic acid, 2-keto acids, and 2-hydroxy acids

Pyruvate is an important chemical intermediate used in the production of pharmaceuticals, agrochemicals, and other fine chemicals. Pyruvic acid can be found in polymers and food additives, while pyruvate esters are used in the cosmetic industry and to make oxygenated "green" solvents. Common pharmaceutical elements synthesized from pyruvate are L-tryptophan, L-tyrosine, alanine, and the Parkinson's drug, levodopa (L-DOPA) (Li *et al.*, 2001). Pyruvate is also used in the making of agrochemicals, including indoxacarb based pesticides (McCann *et al.*, 2001) and plant growth regulator, indol-3-pyruvic acid. Recently, calcium pyruvate has been found to accelerate human fatty acid metabolism (Kalman *et al.*, 1999), suggesting pyruvate for weight loss applications. Besides pyruvate, other 2-keto acids are also very important intermediates or precursors for the synthesis of many drugs and chemicals. As for example, glyoxylic acid is very widely used for the synthesis of vanillin (Esposito *et al.*, 1997).

2-Hydroxy carboxylic acids are largely used in chemical, pharmaceutical and health care industry. (*R*)-Isomer of the first member of optically active 2-hydroxy carboxylic acid, i.e., lactic acid is very expensive compare to its racemic mixture and (*S*)-isomer. (*R*)-Isomer of lactic acid is used as a standard in the laboratory and starting material for the synthesis of chiral compounds (Oikawa *et al.*, 2001). Optically active 2-hydroxy acids are important building blocks for the asymmetric synthesis of wide variety of bioactive molecules such as virus protease and angiotensin converting enzyme (ACE) inhibitors, beta-blockers, A2 antagonists and Ca-channel blockers (Larissegger-Schnell *et al.*, 2006). For example, (*R*)-isomer of 2-Hydroxybutanoic acid derivatives are important building block for the production of a large variety of angiotensin converting enzyme (ACE) inhibitors, which have in common, the (*S*)-homophenylalanine moiety as the central pharmacophore unit (Sheldon, 1993). In another application, 3-phenyllactic acid and its derivatives are frequently used in non-racemic form as components of pharmaceuticals and natural antibiotic agents (Ström *et al.*, 2002). They represent an integral part of bioactive peptides such as

Aeruginosins (Valls *et al.*, 2003) and Microcin (Valls *et al.*, 2002), which are potent protease inhibitors. *p*-Fluoro substituted 3-phenyllactic acid is a key building block for the synthesis of AG7088 (Ruprintrivir), a potent rhinovirus inhibitor used for the treatment of common cold (Tao and McGee, 2002; Dargovich *et al.*, 2002). Optically pure 2-hydroxy acids are used for making chiral synthons like glycols (Prelog *et al.*, 1954), halo esters (Lee and Downie, 1967), and epoxides (Mori *et al.*, 1979). Derivatives of optically active 2-hydroxy acids such as 3-cyclohexyl-2-hydroxy acids are used for making essential component of sialyl Lewis analogs, which are currently in test for treatment of inflammatory disorders (Storz, 2003).

1.2. Pyruvate market analysis

In 2005, the global pyruvate market was estimated at 1,490 tons (pyruvic acid equivalent) with ethyl and methyl pyruvate in highest demand (Avalon Consulting, 2005). Bulk pyruvate cost was found to be 11 USD/kg based on interviews with consumers, suppliers, and agents. This report and cost analysis was used to arrive at a global market value of 15 million USD for pyruvate. The end-use breakdown of pyruvate is shown in Figure 1.

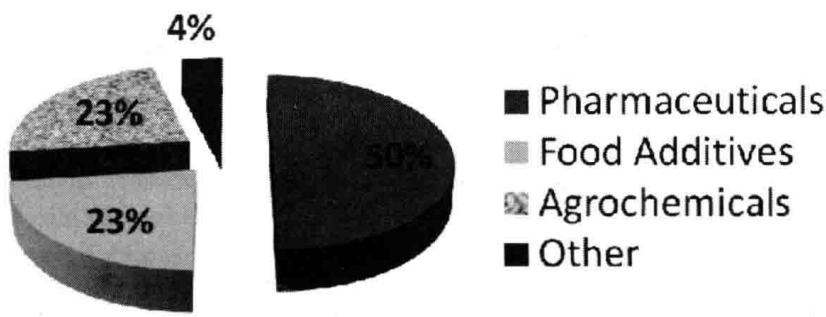

Figure 1. End-use Breakdown of Pyruvate (Avalon Consulting, 2005).

For the market analysis, health supplements or "nutraceuticals" derived from pyruvate has been categorized with other food additives. These food additives, along with agrochemicals, comprise almost one-half of pyruvate usage, while the remaining demand is entirely from the pharmaceutical industry. In pharmaceutics, pyruvate is largely used to synthesize amino acids and other building blocks of more complex drug molecules.

1.3. Methods of preparation of pyruvic acid and chiral 2-hydroxy acids

There are three routes for industrial production of pyruvate: (1) synthesis from tartaric acid (2) direct fermentation and (3) air oxidation of lactic acid. These methods are described below.

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Synthesis from tartaric acid is the oldest method for pyruvate manufacture and is currently being used by at least two commercial plants. This route generates pyruvic acid by thermal decomposition of tartaric acid in the presence of a dehydrogenation agent, such as potassium hydrosulfate (Howard and Fraser, 1932). Thermal decomposition is followed by decarboxylation, and multiple distillations. The overall process consumes extensive energy and results in several undesirable co-products.

Production of pyruvic acid via direct fermentation is an environment friendly alternative to the tartaric acid process. In direct fermentation, an organism is modified to overproduce (or under utilize) pyruvic acid during cellular metabolism (Yonehara and Miyata, 1994). Disadvantages of direct fermentation include low pyruvic acid yield based on glucose input and difficulties associated with product separation from complex fermentation broths. The extent to which this technology is used for commercial production of pyruvate is not known.

Air oxidation route for pyruvate appears to be a superior process. This method boasts 100% lactic acid conversion with single-pass, and pyruvic acid yields of 50 mol% (Ai and Ohdan, 1997). Air oxidation is based on vapor phase oxidative dehydrogenation of lactic acid over an iron phosphate catalyst. This technique is reportedly being adapted for large-scale production of pyruvic acid at Fudan University, China. The economics of this route as well as its commercial status is not available.

Several chemical (Corey *et al.*, 1992) and enzymatic methods (Adam *et al.*, 1995 and 1996) have been published on the synthesis of optically active 2-hydroxy acids. Most reports involve (i) reduction of 2-keto acids with Baker's yeast & lactate dehydrogenase, (ii) the addition of prussic acid to aldehydes by oxynitrilase, (iii) enantioselective oxidation of 1,2-diols with dehydrogenase, (iv) stereoselective esterification of 2-hydroxy acids with lipase, and (v) chemical resolution of racemic 2-hydroxy acids using optically active amines (Sugai and Ohta, 1991; Adam *et al.*, 1997). The disadvantages of these methods include lack of substrate selectivity by enzymes (Adam *et al.*, 1995 and 1996), requirement of expensive co-substrates (Adam *et al.*, 1995 and 1996) or coenzymes (Sugai and Ohta, 1991), requirement of organic solvents (Sugai and Ohta 1991) or the limited availability of some enzymes on a large scale (Adam *et al.*, 1996).

1.4. Glycolate Oxidase for production of pyruvate and chiral (R)-hydroxyacids

Glycolate oxidase (GO) (2-Hydroxyacid oxidase, EC 1.1.3.15) is a flavin mononucleotide-dependent peroxisomal enzyme, which catalyzes the oxidation of 2-hydroxy carboxylic acids such as lactate or glycolate, to the corresponding 2-keto acids (Figure 2). GO is found in many green plants and animals including the leaves of spinach, pea, sugar beet, lettuce, tobacco, pumpkin, cucumber cotyledons, as well

as the liver of pigs, rats and humans (Das *et al.*, 2009). In green plants, glycolate oxidase is involved in photorespiration, a pathway that results in reduced net photosynthesis; while in animals, the enzyme participates in the production of oxalate through metabolic pathways from serine and carbohydrates.

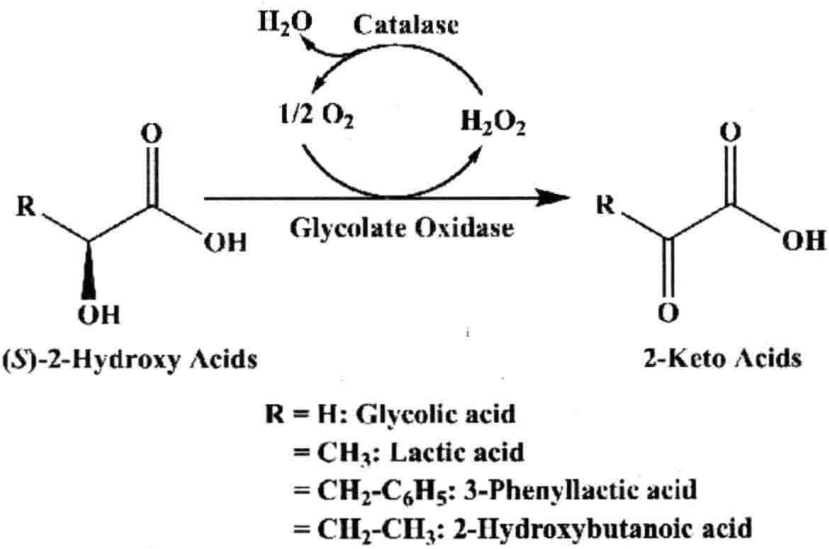

Figure 2. GO mediated conversion of 2-hydroxy acids to 2-keto acids coupled with catalase, which breaks down the co-product hydrogen peroxide to minimize enzyme inactivation and further oxidation of 2-keto acids to byproducts.

The crystal structure of spinach-GO has been published (Lindqvist, 1989). GO was reported to be catalytically active only as tetramers or octamers of identical subunits, with one FMN per subunit (Frigerio and Harbury, 1958). The enzyme activity was also found to be relatively unstable in solution, but the protein tended to irreversibly aggregate into an inactive form, especially in the absence of added FMN. Flavoprotein oxidases catalyze redox reactions in which electrons are transferred from a donor such as 2-hydroxy acids to enzyme-bound flavin (FMN) and, ultimately, to molecular oxygen (Ghisla and Massey, 1989). GO, a flavoprotein oxidase, catalyzes the oxidation of several a-hydroxy acids to their corresponding a-keto acids with stoichiometric production of hydrogen peroxide as co-product (Clagett *et al.*, 1949 and Tolbert *et al.*, 1949). This co-product can deactivate GO or further react with 2-keto acids to yield unwanted byproducts. As a result, catalase was introduced as a second enzyme to break-down hydrogen peroxide (Seip *et al.*, 1993). Figure 2 shows the synergistic roles of GO and catalase in the production of 2-keto acids.

GO from spinach was found to have the highest yield per gram of plant material and highest enzyme activity (Seip *et al.*, 1993). Soluble GO from spinach was combined with catalase T from *S. cerevisiae* to demonstrate the feasibility of an

enzymatic process for the production of pyruvate. Co-immobilization of the enzymes on oxirane acrylic beads increased enzyme stability and product recovery (Seip *et al.*, 1994). However, significant loss of enzyme activity was observed after immobilization. For economical production of pyruvic acid and other 2-keto acids by a combination of GO and catalase, the enzymes need to be cloned in a suitable host such as *Pichia pastoris*. *P. pastoris* has several advantages over *E. coli,* like adaptability to continuous fermentation, high yield of biomass, and expression of proteins in soluble fraction, etc. (Das *et al.*, 2009). In our study, we used double recombinant methylotrophic *P. pastoris* strain NRRL Y-21001 as biocatalyst, where GO from spinach and catalase T from *Saccharomyces cerevisiae* were co-expressed.

In this article, we discuss the production of pyruvic acid, other 2-keto-acids and (*R*)-2-hydroxyacids using the double recombinant *P. pastoris* containing GO and catalase. Pyruvic acid was produced in Kg scale with high purity. Finally, a dynamic process for GO-based production of several (*R*)-2-hydroxy acids in high yield from the corresponding racemic mixtures has been demonstrated.

2. GLYCOLATE OXIDASE BASED PRODUCTION OF PYRUVIC ACID AND OTHER 2-KETO ACIDS INCLUDING 2-HYDROXY ACIDS

2.1. Expression system used in this study

Double recombinant *Pichia pastoris* MSP 8.6 (Payne *et al.*, 1997) used in this study was a gift from DuPont de Nemours and Company to The University of Iowa Research Foundation. The pHIL-D4 expression plasmid (Phillips Petroleum) was used for cloning GO into *P. pastoris* (Payne *et al.*, 1995). The pHIL-D4 plasmid contained a strong methanol-inducible promoter, alcohol oxidase I, (*AOX1*) for expression of the GO gene. A 1110 base-pair DNA fragment encoding spinach GO (Volokita and Somerville, 1987) was sub-cloned into the *EcoRI* site of the pHIL-D4 plasmid, forming pMP1 plasmid. The pMP1 plasmid was cut with restriction enzyme *Sca*I, and the linearized plasmid was used to transform *P. pastoris* GTS115, a His^- host (Phillips Petroleum). Approximately 100 His^+ hosts were screened for reduced growth on methanol (Mut^S) due to disruption of the *AOX1* gene by integration of the GO expression cassette (*AOX1* transplacement). Slow growth on methanol was observed for 17 clones, and these clones were transferred to YPD medium. The YPD was supplemented with increasing concentrations of G418 to screen for high gene copy numbers based on resistance to G418. Two clones were resistant to over 1000 μg G418/mL, so these clones were selected as having the highest gene copy numbers and designated MSP10 and MSP 12.

The 1850 base pair *CTT1* gene encoding for catalase T was obtained by PCR amplification from *S. cerevisiae* DNA. An *Eco*RI site within the *CTT1* gene and lack of a plasmid that was suitable for multiple transformations made construction of

several intermediate plasmids necessary. The resulting plasmid (pMP8) contained the *CTT1* expression cassette following the *AOX1* promoter, *HIS4*, Ap^R, *ori*, and also *SUC2* for selection in hosts previously transformed to His^+. Plasmid pMP8 was cut with restriction enzyme *Dra*III and used to re-transform *P. pastoris* MSP10 that was previously developed for GO production. Plasmid pMP8 was cut at *HIS4* to minimize loss of the GO cassette by adding the *CTT1* cassette at an alternate location in the genome. Transformants were selected by re-plating on sucrose for selection against clones expressing invertase encoded by *SUC2*. PCR examination of the selected transformants showed 41% retained the GO expression cassette, and 89% of those clones had incorporated the *CTT1* cassette to yield double-transformants expressing both GO and catalase T (Payne *et al.*, 1997).

2.2. Production of GO and catalase expressed in P. pastoris

Double recombinant *P. pastoris* expressing GO and catalase was produced in fermenter at 30 L scale. Fermentation conditions are shown in Table 1. For a detailed description of the fermentation procedure, see (Das *et al.*, 2009).

Table 1. Summary of fermentation conditions at 30 L scale

Operating Parameter	**Set-Point**
Organism	Double-recombinant *P. pastoris* MSP 8.6
Temperature	30℃
pH	5.0, controlled with 4 M NaOH
Dissolved Oxygen	30% (calibrated to 100% in air-saturated medium)
Growth	22 h batch growth followed by 12 h fed-batch
Induction	2 – 10 g/L MeOH induction for 28.5 h
OD_{595} @ harvest time	150
Biomass	3.1 Kg of wet weight

Samples at 0, 15, 24, and 28.5 hours post-induction were analyzed by SDS-PAGE to track the expression of GO and catalase. The SDS-PAGE profile including standard molecular weight markers is shown in Figure 3. GO is a tetramer, composed of four subunits, each with a molecular weight of approximately 43,000 Da (Lindqvist and Branden, 1985). For the sample taken before induction, only a faint band of GO was visible at the specified molecular weight. However, the 43,000 Da band became intense as the induction time progressed from 15 to 24, and 28.5 h. Consistent with SDS-PAGE results, GO activity increased throughout methanol induction up to 70 U/g blotted cells measured by DCIP assay (Das *et al.*, 2009) at the final harvest. Catalase is also a tetramer with the molecular weight of each subunit around 60,000 Da (Klei *et al.*, 1990).

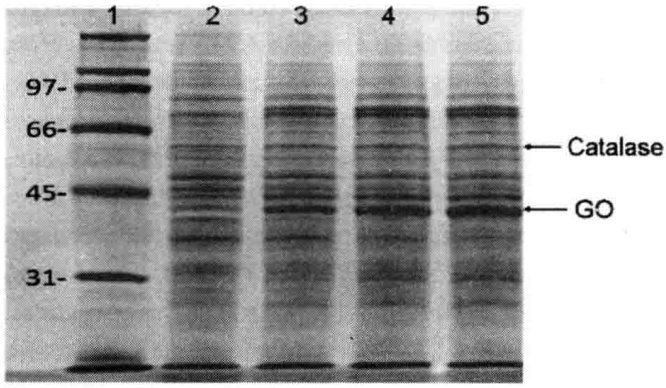

Figure 3. SDS-PAGE profile of GO and catalase expression during methanol induction. Lane Description – Lane 1: Standard protein markers; Lane 2: 0 h post induction; Lane 3: 15 h post induction; Lane 4: 24 h post induction; Lane 5: 28.5 h post induction.

Catalase was present in two forms: a native catalase, which was constitutively expressed, and recombinant catalase T linked to a methanol-inducible promoter. Due to the constant expression of the native catalase, a band at 60,000 Da was visible almost in equal intensity in all samples. Induced vs. native catalase could not be differentiated. The activity by DCIP assay indicated catalase activity of around 125,000 U/g blotted cells at harvest.

2.3. Preparation of GO-biocatalyst for reactions

After the fermentation, cells were harvested by centrifugation and stored at – 80 C until further use. These frozen cells did not lose any activity over a two-year period. Cells as such showed very poor enzyme activity with substrates. So, permeabilization was necessary to make the biocatalyst effective for maximum activities of intracellular enzymes, GO and catalase. Several chemical and physical treatments have been reported for the permeabilization of yeast cells (Felix, 1982). Initial study for permeabilization of *P. pastoris* with lactic acid as a substrate, was carried out using Triton X-100, cetyltrimethylammonium bromide, taurocholic acid, Tween 80, and benzalkonium chloride. Cationinc surfactant, benzalkonium chloride (BC) showed highest activity (Gough *et al.*, 2001). To optimize BC concentration, different 2-hydroxy acids such as lactic acid, 3-phenyllactic acid, and 2-hydroxybutanoic acid were assayed with permeabilized *P. pastoris* at different concentrations of BC. GO and catalase activity of frozen cells without BC-treatment and with 0.02 %, 0.1 % and 0.5 % (w/v) BC-treatment is summarized in the Table 2. The methods of permeabilization have been described in detail (Das *et al.*, 2009). From the Table 2, it is clear that frozen cells without the treatment of BC showed poor activity for both enzymes, as expected. The poor catalytic activities of GO and catalase without permeabilization are probably due to the impermeability of the cells to substrates (Siso *et al.*, 1992). The optimum concentration of BC to achieve maximum GO and catalase activities was, 0.1 %. Lower activity of 0.5 % BC-treated cells could be due to the inactivation of both enzymes (Das *et al.*, 2009).

Table 2. GO activity of permeabilized *P. pastoris* at different concentration of benzalkonium chloride (BC)

BC concentration (w/v)	Average GO activity in IU / g blotted frozen cells			Average Catalase activity in IU / g blotted frozen cells
	Lactic acid	3-Phenyllactic acid	2-Hydroxybutanoic acid	
Frozen cells	17.4	7.8	25.1	42000
0.02 % BC	44.5	13.5	49.4	60000
0.1 % BC	101	25.5	120	123000
0.5 % BC	46.9	11.8	56.0	58000

2.4. GO Activity with different 2-hydroxy acids

GO activity was examined with different 2-hydroxy acids (structures shown in Figure 4) using permeabilized *P. pastoris* whole cells. Glycolic acid, lactic acid, 2-hydroxybutanoic acid, and 2-hydroxy-3-methylbutanoic acid were selected as aliphatics containing short alkyl chain. 3-Chlorolactic acid, and 3,3,3-trifluorolactic acid were chosen as representatives of halogen substituted substrates. 2-Hydroxydecanoic acid was considered as a long chain aliphatic substrate. Mandelic acid, 3-phenyllactic acid and 3-(*p*-hydroxyphenyl)-lactic acid were chosen as aromatic substrates. 3-Indolelactic acid was selected as the heterocyclic substrate. GO and catalase activities were measured by 2,6-dichlorophenol-indophenol (DCIP) assay (Das *et al.*, 2009). DCIP assay results of 2-hydroxy acids are shown in Figure 5. From this figure, it is clear that most of the 2-hydroxy acids were oxidized by GO with the exception of mandelic acid and 2-hydroxy-3-mkethylbutanoic acid. GO exhibited moderate activity with 3,3,3-trifluorolactic acid, 3-phenyllactic acid, 3-(*p*-hydroxyphenyl)-lactic acid, 3-indolelactic acid, and 2-hydroxydecanoic acid. Glycolic acid, lactic acid, 3-chlorolactic acid, and 2-hydroxybutanoic acid were the most active substrates. The rates of oxidation ranged from 1.3 to 120%, relative to the oxidation of lactic acid to pyruvic acid (Das *et al.*, 2009).

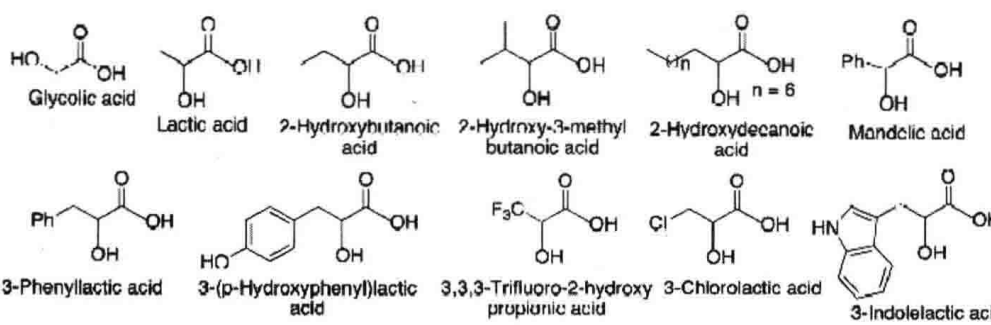

Figure 4. Chemical structures of 2-hydroxy acids used for substrate specificity study. "Ph" indicates Phenyl ring

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