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Studies Directed to the Optimization of Fermentation of Rhodococcus sp. DAP 96253 and Rhodococcus rhodochrous DAP 96622

Gene K. Drago

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by

GENE KIRK DRAGO

Under the Direction of George E. Pierce

ABSTRACT

Bench- and pilot plant scale fed-batch fermentations were performed in stirred-tank bioreactors (STBR) with *Rhodococcus* sp. DAP 96253 and *R. rhodochrous* DAP 96622 in an attempt to elucidate parameters that may affect the optimization of a fermentation process for high biomass production and high inducible expression of cobalt-high-molecular-mass nitrile hydratase (Co-H-NHase. The effects of these factors on amidase (AMDase) activity were also investigated. Biomass and NHase production were inhibited by a total addition of acetonitrile and acrylonitrile (AC / AN) at 500 ppm during a 48 h run. Biomass and enzyme activity were uncoupled when the inoculum mass was increased from 4 g (wet weight) to ≥ 19 g. Other factors that allowed for the uncoupling of biomass production from enzyme activity were the reduction of the AC / AN feed rate from a step-addition at 2500 µl / min to a continuous addition at 80 – 120 µl / min, and the delay to 18 h post-inoculation the time of initial inducer addition. The inhibition of both biomass production and NHase activity was relieved when both the total concentration of AC / AN was reduced to ≤ 350 ppm and the AC / AN feedrate was reduced.

The factors with the greatest influence were shown to be the inducer, the inducer concentration, inoculum mass and source as well as the major carbohydrate and nitrogen source. In addition, this lab is the first to report high AN-specific NHase induction by asparagine (1300 ppm) in a fed-batch fermentation system. Prior to this program, 250 mg of cells (wet weight) per liter could be provided in 4 – 10 days with an activity of 1 U NHase per mg of cells (dry weight). Current production is > 50 g / L in 48 h with an NHase activity > 150 U / mg of dry cell weight.

INDEX WORDS: Amidase, Asparagine, Biodetoxification, Fermentation, Nitrile, Nitrile Hydratase, Rhodococcus

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by

Gene K. Drago

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AC	= acrylonitrile
AMD	= acrylamide
AMDase	= amidase
AN	= acrylonitrile
Asn	= asparagine
BF	= BioFlo® 3000
BTE	= benzene, toluene, ethylene
BTEX	= benzene, toluene, ethylene, xylene
C30	= BioStat® C30-2
Carb	= carbohydrate
cdw	= cell dry weight
COD	= chemical oxygen demand
Co-H-NHase	= cobalt-type high molecular mass nitrile hydratase
CoUrea2nd	= cobalt / urea 2nd addition at time = (x) h
cww	= cell wet weight
dex0to24	= highest dextrose concentration expressed as ppm from t = 0 – 24 h
dex24to44	= highest dextrose concentration expressed as ppm from t = 24 – 44 h
DI	= deionized H_2O
DO	= percent dissolved oxygen
EPDM	= ethylene propylene diene monomer
Fe-NHase	= iron-type nitrile hydratase
GL	= Cargill Dry GL™ 01925
Gln	= glutamine
H : D	= ratio of height to diameter of vessel
H-NHase	= high-molecular-mass nitrile hydratase
HyC	= Hy-Cotton 7803®
In1844hppm	= inducer concentration expressed as ppm from t = 18 – 44 h
Incg	= inoculum mass in grams
IncS	= inoculum state
Ind12hppm	= inducer concentration expressed as ppm from t = 0 – 12 h
Ind18hppm	= inducer concentration expressed as ppm from t = 0 – 18 h
Ind44hppm	= inducer concentration expressed as ppm from t = 0 – 44 h
IndC; Ind conc	= inducer concentration expressed as ppm
IndR; IndRate	= inducer feed / addition rate expressed as µl / min
L-NHase	= low-molecular-mass nitrile hydratase
maltoseR3A	= maltose R3A
MD	= Cottonseed Hydrolysate, Marcor Devolopment Corp.
MG	= Malta Gran® 18M
NHase; NH	= nitrile hydratase

NSB	= net stripper column bottoms
PAH	= polycyclic aromatic hydrocarbon
PB	= phosphate buffer
PBS	= phosphate buffered saline
P-I-D	= proportional-integral-derivative
ppm	= parts per million
Ra	= roughness arithmetic average
RCF	= relative centrifugal force
RTD	= resistance temperature detector
Run Code	= run identification code for internal tracking
SCADA	= supervisory control and data acquisition
SD	= standard deviation
ss	= stainless steel
U / mgcdw	= unit per mg cell dry weight
UPH_2O	= ultrapure H_2O
WWSCB	= wastewater column bottoms
Yd; Yld	= biomass yield
YEMEA	= yeast extract / malt extract

CHAPTER 1 INTRODUCTION

The Genus *Rhodococcus*

The genus *Rhodococcus*, type species *R. rhodochrous* (Zopf 1891) Tsukamura 1974, comprises a heterogeneous group of organisms which has undergone a number of refinements as polyphasic taxonomical approaches have been increasingly employed (Finnerty 1992[15]; Goodfellow 1989[18]; Goodfellow et al. 1998[19]; Gürtler et al. 2004[20]). Currently there are 21 validly described species within the Genus *Rhodococcus* that have the following similar characteristics: they are aerobic, Gram-positive or -variable cocci with sequential, morphological life cycles; they possess cell envelopes that contain sugars such as arabinose and galactose, mycolic acids with 34 – 52 carbons, and dihydrogenated menaquinones with 8 isoprene units (MK-8(H_2)). Rhodococci have been isolated from a variety of environments including temperate and polar soils, and freshwater and marine ecosystems, including the deep sea (Bej et al. 2000[4]; Colquhoun et al. 1998[8]; Mirimanoff and Wilkinson 2000[36]; Uz et al. 2000[48]).

Metabolism

Rhodococci as a group are capable of either Entner-Doudoroff or Embden-Meyerhof-Parnas (in conjunction with the hexose monophosphate pathway) glycolytic pathways, and are known for their ability to transform a variety of complex and/or recalcitrant xenobiotics (Bunch 1998[6]; Finnerty 1992[15]; Warhurst and Fewson 1994[49]). These compounds include, but are not limited to, aliphatic, cyclic, and

polymeric hydrocarbons, halogenated hetero-polycyclic aromatics, aliphatic and aromatic nitriles, and dinitriles. Some of the enzymes involved in these transformations display regio- and stereo-selectivity. Specific examples of the versatile metabolic capabilities of the rhodococci have been reported in the scientific literature. In a study aimed at improving the colonization and subsequent biodegradation of the recalcitrant hydrocarbon polyethylene, Gilan et al. (2004)[17] isolated *R. ruber* C208 from agricultural polyethylene waste. The researchers found that the addition of mineral oil 0.05 % enhanced by 50 % both the colonization and biodegradation of polyethylene by strain C208. The soil isolate *R. rhodochrous* CPC-1 was found to utilize cyclopropanecarboxylate (CPC) as a sole carbon and energy source (Toraya et al. 2004[47]). CPC was used as a model compound for cyclopropane derived herbicides and miticides that may contaminate the environment. The occurrence of cyclopropane moieties in fatty acids among bacteria, plants and yeasts may explain the acquisition of this metabolic pathway. The aliphatic alkanes dodecane and hexadecane were degraded by the psychrotroph *Rhodococcus* sp. strain Q15 at 5 ˚C by both terminal and subterminal oxidation pathways (Whyte et al. 1998[52]). In the presence of alkanes, members of the rhodococci may produce biosurfactants that are mostly cell-bound glycolipids, such as trehalose mycolates or anionic trehalose tetraesters (Lang and Philp 1998[34]; Philp et al. 2002[40]). These biosurfactants make the cell surface hydrophobic and thus able to attach to a layer of alkane making it available for active uptake to inclusion bodies and subsequent degradation (Kim et al. 2002[25]). Halo-substituted alkanes and alkenes also are metabolized by rhodococci, to the extent that this ability is apparently universal.

Rhodococci isolated from UK, Japan, Switzerland, The Netherlands, and US all shared a conserved *dhaA* gene that encodes for haloalkane dehalogenase (Poelarends et al. 2000[42]). 16S rRNA sequences showed < 1 % divergence between haloalkane utilizing strains. In addition, *R. rhodochrous* ATCC 21198 degraded vinyl chloride (from PVC manufacture) after induction of a non-specific oxygenase by isopropanol and acetone, which are degradation products of the normal inducer propane (Kuntz et al. 2003[33]).

In addition to aliphatic alkanes and alkenes, rhodococci possess the ability to degrade aromatic hydrocarbons. Iwabuchi et al. (2002)[22] found rough/smooth phenotypic differences in colony types corresponded to the ability of *R. rhodochrous* S-2 to degrade the aromatic hydrocarbon fraction of an Arabian Light Crude oil. Specifically, only smooth mucoid strains of *R. rhodochrous* S-2 were able to grow on aromatic hydrocarbons as the sole sources of carbon and energy. Mucoid strains of *R. rhodochrous* S-2 produced an extracellular polysaccharide (EPS) of D-glucose, D-galactose, D-mannose, D-glucuronic acid, stearic acid, palmitic acid and oleic acid that emulsified hydrocarbons, making them available for degradation.

Monocyclic aromatic hydrocarbon degradation was investigated by Kim et al. (2002)[24], wherein *Rhodococcus* sp. strain DK17, originally isolated from crude oil contaminated soil, grew on benzene, several alkyl benzenes, phenol, and *o*-xylene as sole carbon and energy sources. It was shown that strain DK17 possessed both *ortho*- and *meta*-cleavage dioxygenases which were induced by benzene; and benzene, toluene, and *o*-xylene, respectively. When the media was diluted to 40 – 100 ppm formaldehyde and 10 – 250 ppm phenol and the packed bed reactor had a 4 h residence time, the reduction

of phenol to below permissible discharge levels and the complete removal of formaldehyde from a phenolic resin manufacturer were demonstrated by *R. erythropolis* UVP-1, which was isolated from a contaminated estuary in Spain (Prieto et al. 2002[43]). As previously described for the aromatic fractions of Arabian Crude Oil, a number of other aromatic compounds are metabolized by the rhodococci. The soil isolate *R. globerulus* PWD1 degraded phenyl-substituted alkanoic acids such as 3-(3-hydroxyphenyl)propionic acid, 3-hydroxyphenylacetate and 3-hydroxycinnamate as the sole source of carbon (Barnes et al. 1997[3]). Also, a *R. rhodochrous* species isolated from a gasoline contaminated aquifer degraded BTEX 1 – 40 ppm (benzene, toluene, ethylene, *o*-xylene, *m*-xylene, and *p*-xylene) and was able to use BTE as sole sources of carbon and energy (Deeb and Alvarez-Cohen 1999[10]). Like the monocyclic compounds, polycyclic aromatic hydrocarbons are substrates for rhodococcal degradative enzymes. Toluene-grown cells of *R. opacus* PWD4, isolated from soil at a gasoline station, regio- and stereospecifically transformed D-limonene to (+) *trans*-carveol (Duetz et al. 2001[14]). Other PAH's transformed by *Rhodococcus* include, biphenyl, polychlorinated biphenyls and naphthalene (Kitagawa et al. 2001[27]; Kosono et al. 1997[32]; Seto et al. 1995[46]; Uz et al. 2000[48]).

An additional issue with fuels/oils besides that of environmental contamination by recalcitrant hydrocarbons is the reduction of sulfur in fuels/oils that contributes to the incidence of acid rain. Diesel oil is typically treated by hydrodesulfurization (HDS) to lower sulfur levels to $\leq$ 500 ppm, with desulfurization between from 500 to 50 ppm being the more desirable yet more difficult target (Kimura et al. 2002[26]). Biodesulfurization

has been used as an alternative to selectively desulfurize hydrocarbons and diesel fuels. *Rhodococcus* sp. strain WU-K2R reduced the content of both benzothiophene 0.27 mM and naptho[2,1-*b*]thiophene 0.27 mM by 57 % and 80 %, respectively, when supplied separately as sole sources of sulfur. *Rhodococcus* sp. strain P32C1 further desulfurized light diesel oil, which was previously desulfurized to 303 ppm by HDS, to 144 ppm (Maghsoudi et al. 2001[35]). Other sulfur containing compounds are also degraded by rhodococci. The thiocarbamate herbicide EPTC (*S*-ethyl dipropylthiocarbamate) at 530 µM was degraded by *R. erythropolis* NI86/21 via a cytochrome P450 (De Schrijver et al. 1997[9]). This strain is also capable of degrading the *s*-triazine herbicide atrazine (2-chloro-4-ethylamino-6-isopropylamine-*s*-triazine).

In addition to the *s*-triazines, reports of other nitrogen-containing compounds metabolized by rhodococci include nitroaromatics from dye, explosive, and pesticide manufacture; as well as amides and nitriles. *Rhodococcus* sp. strain RB1, isolated from activated sludge, was able to mineralize 2,4-dinitrophenol at 1 mM as the sole carbon and nitrogen source (Blasco et al. 1999[5]). Di Geronimo and Antoine (1976)[11] reported an inducible enzyme system which enabled *Nocardia rhodococcus* LL100-21 (subsequently identified as *R. rhodochrous* ATCC 33278) to metabolize 10,000 – 40,000 ppm acetonitrile, butenenitrile, hydroacrylonitrile, propionitrile, succinonitrile, and acetamide each as sole carbon and nitrogen sources. "Aliphatic nitrile hydratase" (NHase) was proposed by Asano et al. (1982)[2] to differentiate, from nitrilase, an inducible enzyme complex in *Arthrobacter* sp. J-1 (*R. rhodochrous* J-1) that was active toward aliphatic nitriles (< 5 carbons) and chloroacetonitrile. Strain J-1 was able to

degrade acetonitrile at 4000 ppm initial concentration within 24 h. NHase activity was stable in the presence of glycerol (50 %) at -20 ˚C for 40 days, and 60 % activity was still retained after 6 months. Purified NHase was not stable when stored at 0 ˚C. Various metal ions such as such as Fe^{+3}, Ni^{+2}, $Mn^{+2,}$ and Ca^{+2} (at 1 mM) had no effect, but the enzyme was competitively inhibited in the presence of KCN 1 mM. The NHase had an aggregate molecular weight of 420 kDa, and was comprised of multiple α and β subunits of 24 and 27 kDa, respectively.

Nitrile Hydratase / Amidase

Two major types of NHase have been characterized based upon the cofactor being either non-heme iron or non-corrin cobalt. In a study by Kashiwagi et al. (2004)[23] with *R. rhodochrous* IFO 15564, DMSO at 20 – 30 % inhibited amidase (AMDase) activity but had little effect on the Fe-NHase activity. Increasing the concentration of the inducer ε-caprolactam from 0 – 4 g / L showed no proportional correlation with total enzyme activity and at > 2 g / L it inhibited growth. Glycerol (80 %) in 0.1 M phosphate buffer (PB) decreased enzyme stability when the enzyme was stored at -20 ˚C and -80 ˚C; while 18 MΩ DI water stabilized activity more than 0.1 M PB.

The molecular weight of *R. rhodochrous* J1 H-NHase (high-molecular mass nitrile hydratase) was shown to be 505 kDa and to be composed of 10 subunits each of 26 kDa (α) and 29 kDa (β), with 1 cobalt atom per α / β subunit pair (Nagasawa et al. 1991[37]; Komeda et al. 1996[29]). $CoCl_2 \cdot 6H_2O$ (10 ppm as Co) was added for the cofactor, and urea 7.5 g / L served as a strong inducer. Organic acids such as: butyric

acid, isobutyric acid, n-valeric acid, and isovaleric acid stabilized enzyme activity. The H-NHase demonstrated broad substrate specificity with aromatic and aliphatic nitriles. The gene order, *nhhC*, *nhhD*, *nhhE*, *nhhF*, *nhhB*, *nhhA*, and *nhhG,* of H-NHase differed from those of other species (Figures 1.1 and 1.2). Komeda et al. (1996)[30] also described in *R. rhodochrous* J1 a L-NHase (low molecular mass NHase) that was induced by $CoCl_2 \cdot 6H_2O$ (10 ppm as Co) and crotonamide 2 g / L. As it was for H-NHase, a gene order was found that was different from other organisms; *nhlD*, *nhlC*, *nhlB*, *nhlA*, and *amdA* (Figures 1.1 and 1.2). A high affinity cobalt transporter coded by *nhlF* was later discovered between *nhlA*, and *amdA* (Komeda et al. 1997[31]).

Fermentation

NHase and AMDase activities as well as biomass may be affected by fermentation parameters such as: the carbon and nitrogen composition of media, dissolved oxygen concentration, inducer, trace elements, and others. Efforts have been made to examine their effects on various nitrile-metabolizing organisms. A batch flask culture of *Brevibacterium imperialis* CBS 498-74 (*Microbacterium imperiale*) was not able to grow with acrylamide (AMD) or acrylonitrile (AN) as sole carbon sources, but with initial concentrations of glucose (5 g / L) and AMD (20 – 30 mM (1600 ppm)) or AN (20 – 30 mM (1600 ppm)), CBS 498-74 obtained its highest NHase activities (Cantarella et al. 2002[7]). Peak NHase activity occurred only after the glucose was consumed from an initial 5 g / L to < 2 g / L. In media optimization experiments conducted by Watanabe et al. (1987)[50], both growth and enzyme activity were

enhanced for *Rhodococcus* sp. strain N-774. They showed that the presence of yeast extract and thiamine·HCl at 20 μg / L promoted higher growth and yeast extract plus $FeSO_4 \cdot 7H_2O$ at 50 mg / L increased NHase activity. When higher levels of biomass were used, higher conversions of AN to AMD were achieved; up to 20,000 ppm AN added. However, when the AN concentration was ≥ 30,000 ppm, conversion to AMD decreased. Although not specifically measured, higher DO led to higher production of enzyme based on the media to flask volume ratio. Both the stability and activity of NHase was higher with intact cells rather than polyacrylamide-immobilized cells. Andale and Hamde (1996)[1] followed the time course of biomass production and NHase activity for *Arthrobacter* sp. C-38 in a series of batch flasks. In a yeast extract malt extract medium (L^-; 15 g glucose, 3 g malt extract, 3 g peptone, and 3 g yeast extract) supplemented with AN at 2000 ppm, the maximum growth was obtained at 60 h. However, NHase activity increased up to 30 h but thereafter decreased. A culture of *Rhodococcus* sp. R 312 (formerly *Brevibacterium*) also exhibited differential time dependence in the biomass and enzyme activity maxima (Ospirian et al. 1999[39]). The maximum activity of the constitutive NHase occurred at 22 h and then declined, while the maximum biomass was achieved after 35 h. The culture, which was supplemented with $FeSO_4 \cdot H_2O$ 20 ppm, $CoCl_2 \cdot 6H_2O$ 200 ppm and glucose 30 g / L, metabolized a wide range of nitriles, including cyanide. In an effort to optimize the culture conditions for increased NHase activity in *P. chlororaphis* B23, a number of parameters were investigated (Yamada et al. 1986[55]). In assessing the effects of iron, the addition of $FeSO_4 \cdot 7H_2O$ (0.001 % w / v) resulted in the greatest increase in Fe-NHase (which showed limited substrate diversity).

Of the seven nitriles and nine amides evaluated, isobutyronitrile was found to be the best nitrile for growth and specific activity, and isobutyramide, crotonamide, and methacrylamide were the best amides for growth and NHase activity. When combined with isobutyronitrile, the best carbon sources for both growth and NHase activity were soluble starch, dextrin and sucrose. Although sucrose was the best sugar for growth, AN with sucrose did not support growth; the AN being inhibitory. The use of maltose at 10 g / L increased growth but not specific enzyme activity, while the use of glucose at 10 g / L had little effect on either growth or enzyme activity. When nitrogen sources were considered, urea (5 g / L) did not increase enzyme activity, and ammonium addition caused nitrogen catabolic repression. Yeast extract and soybean hydrolysate, however, increased growth and NHase activity. A number of amino acids such as L-aspartate, L-cysteine, L-glutamate, and L-proline increased growth and enzyme activity as well. Total NHase produced was 3 % of the total soluble protein with increases of 900X and 90X of enzyme activity and specific enzyme activity, respectively, when compared with their earlier studies.

Commercial Application

Interest in nitrile metabolism has increased in the last few decades because it has been recognized as a promising alternative to synthetic AN and AMD production (Pierce 1999[41]); Weissermel and Arpe 1997[51]). Synthetic production of AN, which is the feedstock for the production of AMD and a variety of other compounds including elastomers, fibers, resins, and thermoplastics, is dominated by the direct ammoxidation of

propene (Sohio process). The world AN production in 1996 was 4.3 million tons (metric). In the Sohio process, propene (or in an improved process propane) is reacted with NH_3, air, and H_2O with a catalyst such as $Bi_2O_3 \cdot MoO_3$ under high temperature (450 ˚C) and pressure (1.5 bar) (Figure 1.2). The reduction of byproducts is attained by the addition of H_2SO_4. The two main waste streams that result from the Sohio process of AN manufacture are wastewater column bottoms (WWCB) and net stripper column bottoms (NSB), which contain a mixture of nitriles, dinitriles, amides, cyanide(s) and ammonium sulfate (Table 1.1) (Wyatt and Knowles 1995[54]). In the U.S., these wastestreams are typically deep-well injected. Concentration ranges of the major organic compounds within the WWCB and NSB wastestreams are listed in Table 1.2 (Pierce 1999[41]).

The synthetic production of AMD generally occurs by acid hydrolysis of AN at 80 – 120 ˚C with the addition of polymerization inhibitors and $(NH_4)_2SO_4$, which results in an expensive separation process. AMD is used to produce polymers for flocculants and petroleum recovery, and had a world demand of 450,000 tons (metric) in 2004 (Nagasawa and Yamada 1995[38]; Will et al. 2005[53]). In contrast to synthetic production methods, biocatalytic methods proceed under milder conditions, generally produce fewer byproducts, and may be more regio- and stereoselective (Pierce 1999[41]). *R. rhodochrous* J1, a strain with high nitrile hydratase NHase activity, has been used by the Nitto Chemical Industry, Ltd. to commercially produce acrylamide from AN at atmospheric pressure and 20 ˚C with yields up to 20,000 tons / yr (Weissermel and Arpe 1997[51]). These yields are within the range of synthetic AMD plants and occur at nearly 100 % conversion.

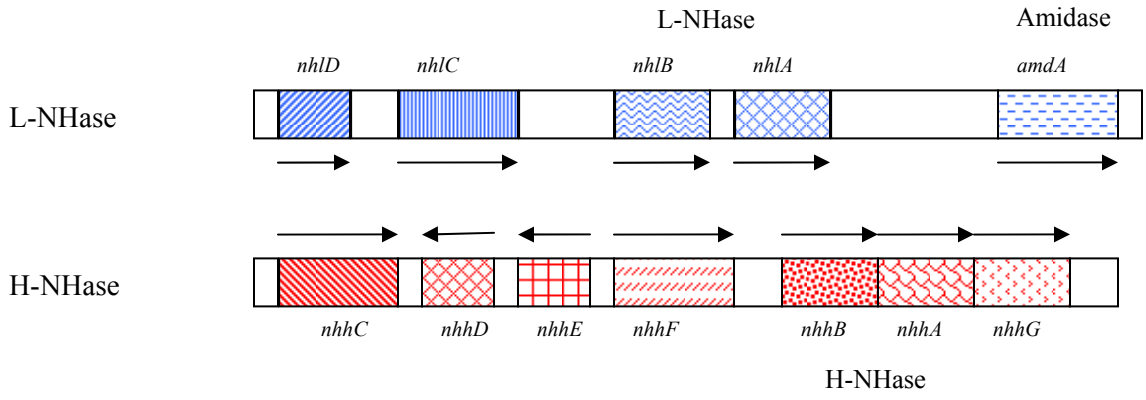
As it is unlikely that biocatalytic processes will surpass synthetic AMD production in the near future, research on biological methods that reduce the environmental impact of nitrile production wastewater continues to be the vanguard. A defined mixed culture, some of which included: *Acinetobacter* sp., *Alcaligenes* spp., *Bacillus megaterium*, *Flavobacterium* sp., *Klebsiella pneumoniae*, and *Pseudomonas* spp., degraded wastewaters from a nitrile manufacturing plant (Wyatt and Knowles 1995[54]). In the Wyatt and Knowles (1995) experiment, the major organic compounds in the WWCB included: acetaldehyde 28 ppm, acrolein 52 ppm, acetic acid 1310 ppm, acrylic acid 1827 ppm, acrylamide 821 ppm, cyanopyridine 620 ppm, fumaronitrile 794 ppm, maleimide 1818 ppm, and succinonitrile 231 ppm. The major organic compounds in the NSB included: acetaldehyde 5 ppm, acrolein 36 ppm, acetic acid 2323 ppm, acrylic acid 786 ppm, acrylamide 49 ppm, cyanopyridine 65 ppm, fumaronitrile 66 ppm, maleimide 91 ppm, and succinonitrile 2380 ppm. The concentration of AN was not provided for either wastestream. The NSB was amended with minimal salts plus phosphate buffer, and the pH was maintained at ~ pH 7.2 with H_2SO_4. Batch fermentations (1L) were switched to continuous mode and ran 800 – 2400+ h. Degradation of the target compounds was measured by chemical oxygen demand (COD), and the NSB influent and effluent COD's were ~24,500 and 5500 ppm, respectively. The WWCB was diluted 1:10 before use because of its toxicity. The culture then was able to mineralize 75 – 80 % of the original COD. Greater COD removal was achieved when condensates of distilled WWCB (80 – 85 % COD removal) and NSB (90 % COD removal) were used as influents. The members of mixed cultures, however, are difficult

to maintain as consistent metabolically specialized subpopulations, especially as the wastestream from the Sohio process may vary in the concentrations of substrates. Pure cultures that were capable of degrading the mixtures of nitriles found in the Sohio process wastestream was achieved by Pierce (1999)[41]. *Rhodococcus rhodochrous* strain DAP 96622 and *Rhodococcus* sp. strain DAP 96253, when multiply induced for NHase, were capable of treating production wastestream from the Sohio process that may contain AN 5 – 1250 ppm and AC 0 – 4500 ppm. Degradation did not require oxygen, and was done by actively growing, immobilized, and resting cells (cells that had been stored at 4 ˚C x 6 – 60 d). The NHase activity was not inhibited by cyanide at 50 ppm or ammonium sulfate (0 – 8 % wt / wt). Conditions were generally defined for the growth of the cultures and induction of H-NHase activity. These included the use of Bacto™ R2A (BD, Sparks, MD) or YEMEA as growth media, cobalt 1 – 25 ppm, and urea 1 – 10 g / L, AC 150 ppm and AN 150 ppm, and succinonitrile 50 ppm as inducers.

Hypothesis

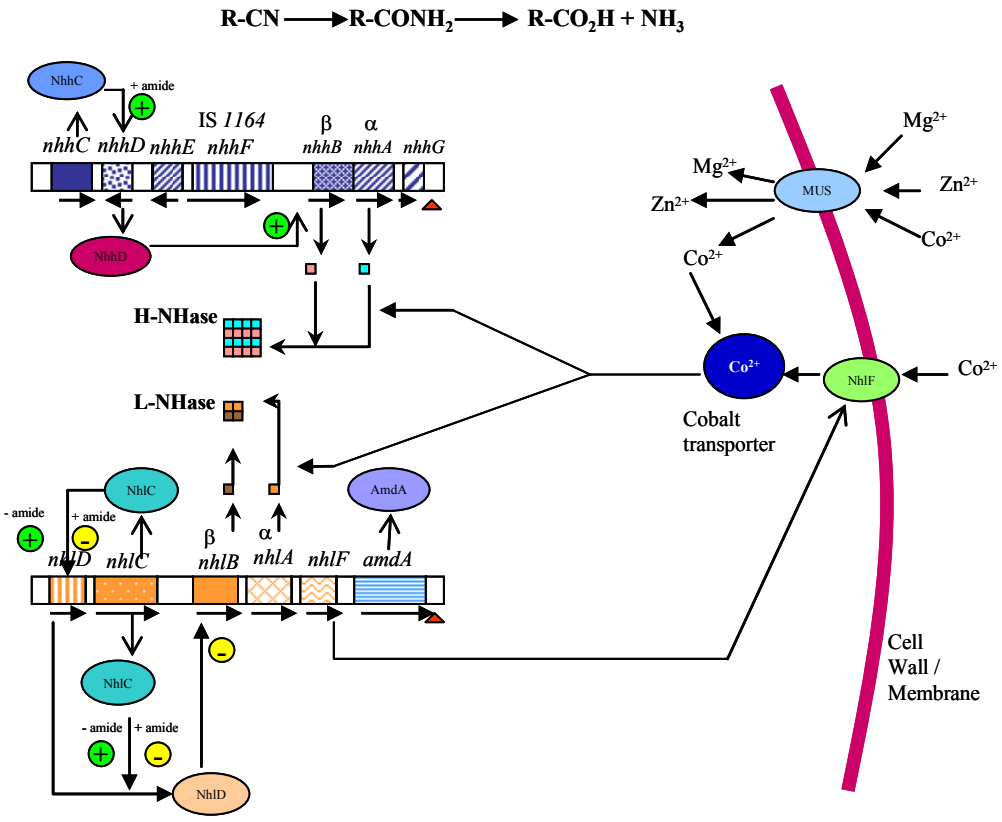
Based upon the previously described research on fermentations and NHase induction in rhodococci and other strains, it is hypothesized that nutrient and inducer concentrations will affect both the capacity to increase biomass production and inducible expression of cobalt-high-molecular-weight nitrile hydratase (Co-H-NHase) of *Rhodococcus rhodochrous* strain DAP 96622 and *Rhodococcus* sp. strain DAP 96253. In addition, it is hypothesized that scoping experiments will identify unanticipated variables that will allow for improved biomass and enzyme activity.

Figure 1.1 Gene Order of L-NHase and H-NHase.



Adapted from Komeda et al. 1996[30].

Figure 1.2 Gene Regulation of L-NHase and H-NHase



Adapted from Kobayashi, M. and S. Shimizu. (1998)[28].

Table 1.1 Representative Components of Acrylonitrile (AN) Wastewater

	ppm	
Component	NSB	WWCB
Ammonia nitrogen	160	22,000
Kjeldhal nitrogen	180	46,600
Total organic carbon	4,300	11,000
Chloride	3	100
Dissolved solids	3,880	98,100
Suspended solids	7	140
Sulfate	25	76,000
Cyanide total	450	1,000
pH	4.7	5.8

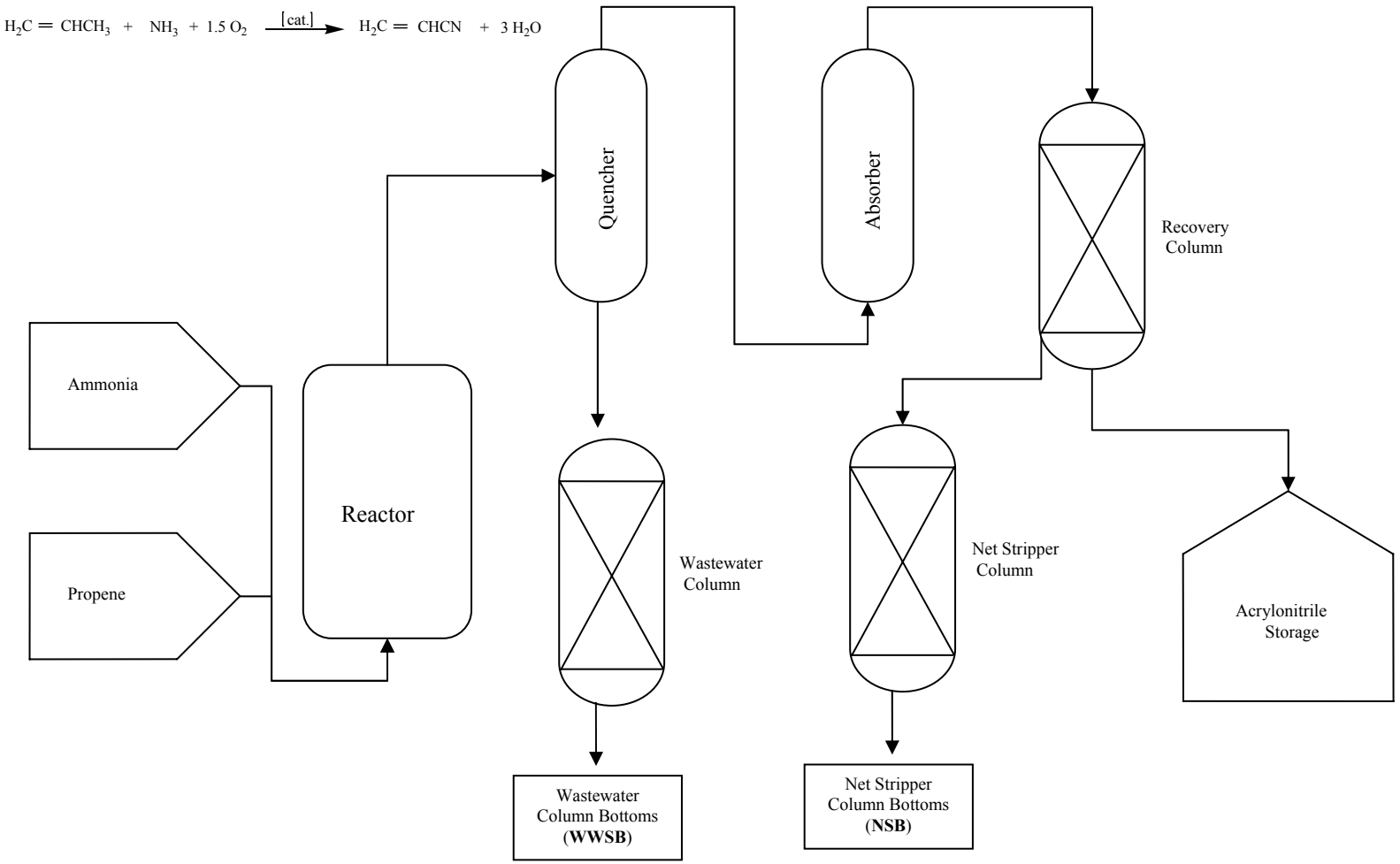
Adapted from Wyatt and Knowles (1995)[54].

Table 1.2 Concentration Ranges of Organic Compounds Found in Representative Acrylonitrile Wastewater

	ppm	
Compound	NSB[a]	WWCB[b]
Acrolein	10 – 100	50 – 1200
Acrylamide	10 – 130	1100 – 1500
Proprionitrile	≤ 20	10 – 150
Acetonitrile	0 – 3000	20 – 4500
Acrylonitrile	5 – 180	10 – 1250
Succinonitrile	300 – 40,000	50 – 5000
Fumaronitrile	≤ 100	20 – 1500

[a]NSB = Net Stripper Bottom
[b]WWCB = Wastewater Column Bottom
Adapted from Pierce (1999)[41].

Figure 1.2 Basic Schematic of Acrylonitrile (AN) Production / Ammoxidation of Propene



CHAPTER 2 MATERIALS & METHODS

Chemicals / Media / Reagents

Chemicals, media components, and reagents were from the following manufacturers and suppliers: Becton Dickinson, Sparks, MD; Cargill Inc., Minneapolis, MN; Difco Laboratories Detroit, MI; Eastman Kodak Co, Rochester, NY; EM Science, Gibbstown, NJ; J.T. Baker Chemical Co., Phillipsburg, NJ; Fisher Scientific Co., Fairlawn, NJ; Mallinckrodt Chemical Inc, Paris, NY; Marcor Development Corp., Carlstadt, NJ; Primera Foods Corp., Cameron, WI; Quest International, Hoffman Estates, IL; Sigma-Aldrich Corp., St Louis, MO; and Trader's Protein, Memphis, TN.

Culture

Rhodococcus sp. strain DAP 96253 was derived from *Rhodococcus* sp. strain ATCC 39484, which was isolated from chemical waste, and *R. rhodochrous* strain DAP 96622 was derived from *Rhodococcus rhodochrous* (Zopf) Tsukamura emend. Rainey et al. (ATCC 33278). Both strains DAP 96253 and DAP 96622 were characterized by an ability to detoxify various nitriles and amides when induced (Pierce 1999[41]).

Vessels / Control

Fermentation runs were performed in a BioFlo® 3000 Benchtop Fermentor (NBS; New Brunswick Scientific Company, Inc., Edison NJ) and a BioStat® C30-2 (Sartorius BBI Systems, Melsungen, Germany). The BioFlo® 3000 consisted of a 14 L total volume (10 L working volume) and ~ 2 : 1 height to diameter (H:D) aspect ratio glass vessel with

side-wall penetrations, and a stainless steel (ss) head plate. Penetrations were provided in the head plate for installation of a direct-drive top-mounted DC motor, the sensing probes (dissolved oxygen (DO), foam, pH, resistance temperature detector (RTD)), gas ring-sparger, the water-cooled exhaust condenser, and various addition ports. Each connection was made air- and water-tight with ethylene-propylene diene monomer (EPDM) o-rings. The inoculation septum was also of EPDM. On the impeller shaft were installed 2 Rushton impellers, or 1 Rushton (top) and 1 marine blade impeller (bottom). The impeller shaft passed through a single mechanical seal that served as an aseptic connection within the bearing housing. A baffle assembly was installed that consisted of four ss baffles welded to a ss support frame. The pH was measured with a 325 mm FermProbe® pH electrode (Broadley-James Corp., Irvine, CA) that was calibrated prior to installation, and the pH was adjusted with sterile 1N or 2N H_2SO_4 and 1N or 2N NaOH. DO was measured with a 320 mm polarographic OxyProbe® (Broadley-James Corp., Irvine, CA) that was calibrated following sterilization with 100 % N_2 for zero and 100 % O_2 for span; and temperature was measured with a platinum Pt-100 (RTD). Deviations from the user-defined setpoints for pH, DO and temperature were each corrected via proportional-integral-derivative (P-I-D) control. A conductivity foam probe installed in the head plate and a dedicated pump connected to an addition bottle that contained sterile antifoam provided foam control. Inlet and exhaust gases were filtered through separate 0.2 μm sterile filters. The vessel was sterilized by autoclave with all probes installed except for the Pt-100, and all open ports were hooded with foil. After sterilization, the motor was attached, glycerin was added to the thermowell and the RTD was installed.

Also, the tubing for additions, heating and cooling water, the inlet air, and cables for the probes were connected. Supervisory control and data acquisition (SCADA) software, *AFS*-BioCommand® Bioprocessing Software Version 2.62 (NBS, Edison, NJ), provided event and time-based control algorithms for the run parameters.

The BioStat® C30-2 (Sartorius BBI Systems, Melsungen, Germany) had a 42 L total volume (30 L working volume) and 2.2 : 1 H:D aspect ratio ss vessel with the interior finish electropolished to Ra = 0.8 μm. The vessel had upper and lower side-wall penetrations, as well as a penetration in the bottom dish for the harvest/drain valve. The lower side-wall penetrations were for installment of the DO, pH and RTD probes and the sampling valve; while the upper side-wall penetrations included spare ports, an inoculation valve port, a safety rupture disc, and connections to two ss heat exchangers for temperature regulation. The vessel and piping were supported by a ss open frame. The ss head plate had penetrations for inoculation and addition ports, inlet air to ring sparger, foam and level probes, exhaust condenser fitted with a high foam probe, a direct-drive top-mounted 900 W brushless motor, and a lighted viewing port. The impeller shaft was fitted with 3 Rushton impellers and a single mechanical seal. Four removable ss baffles were installed in the vessel. Wetted o-rings and septa were of EPDM, silicone, and polytetrafluoroethylene (PTFE). The pH was measured with a 120 mm DPAS probe (Mettler-Toledo Ingold, Inc., Bedford, MA) that was calibrated prior to installation in the vessel wall, and the pH was adjusted with 1N or 2N H_2SO_4 and 1N or 2N NaOH. DO was measured with a 120 mm Ingold® 6100 polarographic probe (Mettler-Toledo Ingold) that was calibrated, following sterilization, with 100 % N_2 for zero and 100 % O_2 for

span; and temperature was measured with a platinum probe Pt-100 (RTD). Deviations from the user-defined setpoints for pH, DO and temperature were each corrected via P-I-D control. The foam, high foam, and level probes were of the conductivity type. Inlet and exhaust gases were filtered through 0.2 μm steam-sterilized filters encased in ss housings. Prior to sterilization, all ports were closed or plugged and all probes were installed. The vessel was sterilized-in-place by a dedicated steam generator that provided plant and clean steam through ss filters. Following sterilization, multi-channel addition ports with attached sterile addition tubing were inserted into the head plate through EtOH-flamed septa. SCADA software was MultiFermentor Control System (MFCS) Version 2 (Sartorius BBI Systems).

Base Medium

The base medium constituted 1 or 1.5X R2A or R3A (Reasoner and Geldreich 1985[44]). Following sterilization of the BioStat® vessel that contained 8.5 L (9.4 L actual to account for evaporative losses) ultrapure Type I reagent grade H_2O (UPH_2O), sterile concentrated R3A was aseptically transferred to the vessel. The BioFlo® was autoclaved with 1.5 L UPH_2O, and R2A or R3A and make-up UPH_2O were aseptically transferred.

Corrective Measures / Inducer Addition / Supplementation

For induction of H-NHase; at t = 0 h, sterile $CoCl_2 \cdot 6H_2O$ and urea were added to achieve 50 ppm $CoCl_2$ and 7500 ppm urea. An equivalent volume of urea and 150 ppm

$CoCl_2$ were added at 4 – 6 or 24 – 30 h. In addition to the urea, 300 – 500 ppm (final concentration) AC / AN or 0.1 M – 0.2 M Asn were added step-wise or at a constant rate that began at various times. A feed profile for nutrient supplementation was set to gradually replace the R2A or R3A base medium with a richer medium, 2X YEMEA (Dietz and Thayer 1980[12]). Other nutrient supplements included maltose 50 % (w / v) and dextrose 50 %.

Inoculum

Inocula were prepared from cultures incubated ~ 7 d at their respective growth temperatures (DAP 96253 = 30 ˚C, DAP 96622 = 26 ˚C) on YEMEA plates with a selected inducer (approximately 10 plates per L). Alternatively, inocula were prepared from frozen 20X cell concentrates from previous runs revived in R2A or R3A and incubated shaking at their respective growth temperatures. Thirdly, inocula were grown in biphasic YEMEA media. The inocula from the plates and biphasic media were harvested, and washed and re-suspended in phosphate buffer at 50 mM for immediate use or stored O / N at 4 ˚C. The inocula from frozen cells were decanted into sterile addition bottles and then transferred to the vessel.

Sampling

From the BioFlo®, sampling for off-line measurements of glucose concentration and enzyme activities, and for verification of culture stability was done via a sterile syringe connected aseptically to an installed ss tube. A manually actuated re-sterilizable

Keofitt® radial diaphragm valve was fitted into a 90˚ 25 mm lower side-wall port in the C30-2.

Glucose monitoring

A YSI 2700S (Yellow Springs Instruments, Inc., Yellow Springs, OH) automatically sampled and measured D(+) glucose concentrations with a Ag / Pt polarograhic probe every 1.2 – 1.5 h. Automatic sampling by the YSI was made via aseptic connections. During various runs, the YSI was set to correct for glucose concentrations < 1.00 g / L by adding dextrose 50 % or maltose 50 % via P-I-D control of an externally connected pump (Masterflex® Console Model 77521-50, Cole-Parmer Instrument Co., Vernon Hills, IL).

Enzyme Analysis

Activities of NHase, and AMDase were measured by substrate reduction and product formation via a gas chromatograph, or by evolution of ammonia via a spectrophotometer. Method details were previously reported (Ganguly 2006[16]).

Harvest

The runs were discontinued at ~ 48 h, and the culture in the BF 3000 was harvested with sterile Tygon® tubing and a filling bell aseptically connected from the harvest tube to sterile 2 L media bottles. The exhaust port was plugged and a low-volume of inlet gas forced the culture into the receiving bottles. The culture in the C30-2

was aseptically transferred to sterile receiving bottles via sterile tubing and a filling bell for batch centrifugation, or pumped directly to a centrifuge for continuous centrifugation. Batch centrifugations were performed with a Sorvall RC5B Plus fitted with a GSA rotor, Kendro Laboratory Products, Newtown, CT, and an Avanti XPI J-20 fitted with a JA-10 rotor, Beckman Coulter, Inc., Fullerton, CA at a relative centrifugal force (RCF) = 4,785, 10,409 or 13,000 at 4 ˚C x 10 – 15 min. The supernatant was decanted and the wet weight of the pellet was determined. Dewatering was performed by continuous centrifugation with a Carr Powerfuge® Pilot (Pneumatic Scale Corp., Cuyahoga Falls, OH) operated at RCF ~ 10,409 or 13,000 with feed rates between 100 and 500 ml / min.

Storage

Pelleted cells were resuspended to a 20X concentrated volume in phosphate buffered saline (PBS) at 50 mM supplemented with the inducer used during the fermentation, dispensed to sterile polypropylene tubes, and flash frozen in dry ice and acetone. The frozen, concentrated cells were then stored at either -20 or -80 ˚C.

Statistical Analysis

Statistical analyses were performed with SPSS® 12.0 (SPSS Inc., Chicago, IL). Hierarchical cluster analysis was performed by between-group linkage with squared Euclidian distances of selected variables. All correlations reported are Spearman's rank correlation coefficients.

CHAPTER 3 RESULTS

Table 3.1 shows a listing of those variables or parameters which were evaluated for their effect on biomass, NHase, and AMDase in order to identify those variables that may contribute to the optimization of a fermentation process for high biomass production and Co-H-NHase induction of *R. rhodochrous* strain DAP 96622 and *R.* sp. DAP 96253. The frequencies with which these variables were employed are shown in Tables 3.2 A – K. The majority of the 58 runs were performed in the BioFlo® 10 L (BF) before the attempted scale-up to the BioStat® 30 L (C30) (Table 3.2 A). Although *Rhodococcus* sp. DAP 96253 and *R. rhodochrous* DAP 96622 are distinct *Rhodococcus* species, they were both capable of obtaining high biomass and NHase activities and low AMDase activities. DAP 96253, which is less susceptible to contamination (i.e. less fastidious) was chosen for the majority of all runs (Table 3.2 B). Runs with DAP 96622 were performed only after trends were established using DAP 96253. SPSS 12.0 statistical analysis software was used to construct a hierarchical cluster analysis of the variables biomass yield (g / L cww); and NHase and AMDase activities in U / mgcdw, with 1 U = amount of enzyme necessary to convert 1 μmol C≡N to NH_2 per min. Druzhinina et al. (2006)[13] used cluster analysis to explore patterns in carbon utilization and growth of the ascomycete *Hypocrea jecorina*. In the current study, Spearman's rank correlation coefficient was used to further assess the relationship between the non-normally distributed data and variables with ordered codes. The Pearson correlation coefficient is not valid for such data because it assumes that the data is normal, and quantitative. Correlations quantify the relationship between two variables by taking into account the difference in means for

each variable as well as the sample number and standard deviations. Figure 3.1 provides a hierarchical cluster analysis in which two major clusters formed. In the first major cluster, there was significant correlation with low to very high biomass yields (15 – 88 g / L) and relatively low NHase (28 – 91 U / mgcdw) and low AMDase (0 – 5 U / mgcdw) activities that were all run in the C30 (Cluster I, C30). The second major cluster separated distinctly into two sub-clusters of runs with high to very high biomass yields (33 – 74 g / L), high to very high NHase activities (155 – 180 U / mgcdw), and low to medium AMDase (2 – 8 U / mgcdw) activities that were all performed in the BF (Sub-cluster II*b*, BF); and runs with low to high biomass (8 – 53 g / L), medium to very high NHase activities (109 – 162 U / mgcdw), and low to high AMDase activities (2 – 25 U / mgcdw), performed in both the BF and C30 (Sub-cluster II*a*, Mixed Vessel Cluster). These clusters are also highlighted in Table 1.

For the mixed vessel cluster, Spearman's rho correlation coefficients were calculated for biomass yields. The correlations between biomass yields and NHase and AMDase activities were negative at the 0.01 confidence level and the 0.05 confidence level, respectively. When biomass yield was low or high, the corresponding NHase and AMDase activities were high or low, respectively. Variables/Parameters that affected yield were found to vary by vessel and inducer. In Figure 3.2, the AC / AN induced runs in the BF had mean biomass yields significantly lower than not only those biomass yields from AC / AN induced runs in the C30, but also lower than those biomass yields from Asn induced runs for either vessel.

Table 3.1 Summary of Parameters / Variables Evaluated

Run Code	Vessel	Strain	Nitrogen	Carb	Inducer	Ind conc	Indl2hppm	Indl8hppm	Indl844hppm	Ind44hppm	IndRate	NHase	AMDase	CoUrea2nd	DMY	Yield	dex0to24	dex24to44	IncS	Incg
Run12	BF	96253	PP3	dex+maltose	none	9	9999	9999	9999	9999	9999	999	999	4-6h	none	9	.	.	Fr	999
Run13	BF	96253	PP3	dex+maltose	none	9	9999	9999	9999	9999	9999	999	999	4-6h	none	19	.	.	plate	28
Run14	BF	96253	PP3	dex+maltose	none	9	9999	9999	9999	9999	9999	999	999	4-6h	none	6	.	.	plate	16
Run21	BF	96253	PP3	dex+maltose	none	9	9999	9999	9999	9999	9999	999	999	4-6h	none	3	.	.	Fr	999
Run23	BF	96253	PP3	dex+maltose	none	9	9999	9999	9999	9999	9999	999	999	4-6h	none	13	.	.	plate	20
Run33	BF	96253	Pharmamedia	dex+maltose	AC/AN	500 ppm	200	300	200	393	2500	18	999	4-6h	none	1	2	4	plate	4
Run34	BF	96253	PP3	dex+maltose	AC/AN	500 ppm	200	300	200	393	2500	130	999	4-6h	none	4	2	3	plate	20
Run36	BF	96253	PP3	dex+maltose	AC/AN	500 ppm	200	300	200	393	2500	999	999	4-6h	none	3	.	.	plate	20
Run38	BF	96253	PP3	dex+maltose	AC/AN	500 ppm	200	300	200	393	2500	999	999	4-6h	none	1	.	.	Fr	2
Run39	BF	96253	PP3	dex+maltose	AC/AN	500 ppm	200	300	200	393	2500	999	999	4-6h	none	5	.	.	Fr	5
Run310	BF	96253	PP3	dex+maltose	AC/AN	500 ppm	140	206	204	346	80	999	999	4-6h	none	4	4	7	Fr	9
Run311a	BF	96253	Proflo	dex+maltose	AC/AN	500 ppm	140	206	204	346	80	999	999	9	none	17	4	6	plate	999
Run312	BF	96253	PP3	dex+maltose	AC/AN	500 ppm	140	206	204	346	80	999	999	4-6h	YEMEA	1	.	.	Fr	11
Run314	BF	96253	HyC	dex+maltose	AC/AN	500 ppm	140	206	204	346	80	999	999	4-6h	none	19	.	.	plate	33
Run316	BF	96253	HyC	dex+maltose	AC/AN	500 ppm	220	308	380	600	120	999	999	4-6h	none	14	.	.	plate	32
Run318	BF	96253	HyC	dex+maltose	AC/AN	500 ppm	220	308	380	600	120	999	999	4-6h	none	11	.	.	plate	30
Run321	BF	96253	HyC	dex+maltose	Asn	0.2 M	288	429	560	962	120	168	2	4-6h	none	36	2	1	plate	30
Run322	BF	96253	HyC	dex+maltose	Asn	0.2 M	140	345	770	1050	120	155	6	20-28h	YEMEA	52	1	0	plate	30
Run323	BF	96253	HyC	dex+maltose	AC/AN	500 ppm	72	172	382	529	120	162	3	20-28h	none	8	3	6	plate	32
Run325	BF	96253	HyC	dex+maltose	AC/AN	350 ppm	50	120	268	371	120	170	5	20-28h	YEMEA	38	1	0	plate	30
Run42	BF	96622	HyC	dex+maltose	Asn	0.1 M	0	0	1850	1850	2500	172	2	20-28h	none	44	1	0	plate	30
Run43	BF	96622	HyC	dex+maltose	Asn	0.1 M	0	0	1770	1770	2500	166	5	20-28h	none	39	1	0	plate	30
Run45	BF	96622	HyC	dex+maltose	AC/AN	500 ppm	0	0	600	600	2500	161	6	20-28h	none	14	1	0	plate	30
Run46	BF	96622	Proflo	dex+maltose	AC/AN	500 ppm	0	0	164	164	50	143	9	20-28h	none	14	4	8	plate	30
Run47	BF	96622	MD/MDUF	dex+maltose	Asn	0.1 M	0	76	1310	1360	2500	165	2	20-28h	YEMEA	57	1	1	plate	19
Run48	BF	96622	MD/MDUF	dex+maltose	Asn	0.1 M	0	0	2020	2020	2500	125	2	20-28h	dex	42	2	0	plate	30
Run49	BF	96622	MD/MDUF	dex+maltose	AC/AN	350 ppm	50	120	268	371	120	178	6	20-28h	dex	58	1	8	plate	30
Run410	BF	96253	MD/MDUF	dex+maltose	AC/AN	350 ppm	50	120	268	371	120	180	8	20-28h	dex	34	2	10	plate	30
Run411	BF	96253	MD/MDUF	dex+maltose	AC/AN	350 ppm	50	120	268	371	120	175	8	20-28h	dex	51	1	3	plate	30
Run412	BF	96253	MD/MDUF	dex+maltose	Asn	0.15 M	138	330	896	1160	150	175	8	20-28h	dex	59	1	0	plate	999
Run413	BF	96253	MD/MDUF	dex+maltose	AC/AN	350 ppm	13	85	268	342	120	170	3	>28h	dex	47	4	2	plate	27
Run415	BF	96253	HyC	dex+maltose	Asn	0.15 M	138	330	723	1000	120	171	4	20-28h	dex	74	5	2	Fr	66
Run416	BF	96253	HyC	dex+maltose	AC/AN	350 ppm	50	120	268	371	120	170	2	20-28h	dex	66	.	.	plate	22
Run417	BF	96253	HyC	dex+maltose	AC/AN	350 ppm	50	120	268	371	120	168	2	20-28h	none	33	.	.	plate	22
Run418	BF	96253	HyC	dex+maltose	AC/AN	350 ppm	50	120	268	371	120	150	8	20-28h	dex	22	7	10	Fr	66
Run421	BF	96622	HyC	dex+maltose	Asn	0.15 M	110	265	580	980	120	132	2	>28h	none	37	4	3	Fr	8
Run423	C30	96253	HyC	dex+maltose	Asn	0.15 M	590	850	1000	1730	450	136	4	20-28h	none	45	.	.	Bp	999
Run424	C30	96253	HyC	dex+maltose	Asn	0.15 M	9999	9999	9999	9999	625	128	2	>28h	none	47	.	.	Bp	999
Run428	C30	96253	MD/MDUF	dex+MG	AC/AN	300 ppm	0	0	280	280	1000	140	14	20-28h	none	27	.	.	Bp	999
Run429	C30	96253	MD/MDUF	dex+MG	AC/AN	300 ppm	0	0	257	257	666	119	4	20-28h	none	23	.	.	Bp	999
Run430	C30	96253	MD/MDUF	dex+GL	AC/AN	350 ppm	19	45	219	255	350	122	9	20-28h	none	53	.	.	Bp	999
Run431	C30	96253	MD/MDUF	dex+GL	AC/AN	350 ppm	59	140	185	300	500	137	2	20-28h	none	45	.	.	Bp	999
Run53	C30	96253	MD/MDUF	GL	Asn	0.15 M	440	1000	1900	2700	1000	86	1	20-28h	none	52	.	.	Fr	140
Run55	C30	96253	MD/MDUF	GL	Asn	0.15 M	440	1000	1900	2700	1000	72	5	20-28h	none	15	.	.	Bp	999
Run56	C30	96253	MD/MDUF	dex+GL	Asn	0.15 M	0	0	2100	2100	3300	69	2	20-28h	none	50	.	.	Bp	999
Run58b	BF5L	96253	MD/MDUF	maltoseR3A	Asn	0.15 M	110	265	3290	3500	126	123	7	20-28h	none	38	.	.	Bp	5
Run59	C30	96253	MD/MDUF	maltoseR3A	Asn	0.15 M	221	620	2000	2700	1000	159	22	20-28h	none	16	.	.	Bp	14
Run510	C30	96253	MD/MDUF	maltoseR3A	Asn	0.15 M	221	620	2000	2700	1000	122	20	20-28h	none	32	.	.	Bp	18
Run512	C30	96253	HyC	maltoseR3A	Asn	0.15 M	0	255	2100	2250	870	109	25	20-28h	none	20	3	0	Bp	15
Run513	C30	96253	HyC	maltoseR3A	Asn	0.15 M	9999	9999	9999	9999	400	139	2	20-28h	none	33	3	0	Bp	15
Run514	C30	96253	HyC	maltoseR3A	Asn	0.15 M	210	500	1000	1420	476	137	6	20-28h	none	35	.	.	Bp	15
Run515	C30	96253	HyC	maltoseR3A	Asn	0.15 M	210	500	1000	1420	476	53	0	20-28h	maltose	40	.	.	Bp	16
Run516	C30	96253	HyC	maltoseR3A	Asn	0.15 M	210	500	976	1350	476	76	3	20-28h	maltose	40	1	0	Bp	15
Run517	C30	96622	HyC	maltoseR3A	Asn	0.15 M	440	1000	2000	2790	1000	114	4	20-28h	none	43	2	0	Bp	19
Run518	C30	96622	HyC	dex+maltose	Asn	0.15 M	9999	9999	9999	9999	750	57	1	20-28h	dex	43	0	7	Bp	17
Run519	C30	96253	HyC	dex+maltose	Asn	0.15 M	0	0	1800	1800	1000	28	5	20-28h	dex	40	6	6	Bp	19
Run520	C30	96253	HyC	dex+maltose	Asn	0.15 M	440	1000	1910	2650	1000	88	1	20-28h	dex	63	4	3	Bp	18
Run521	C30	96253	HyC	dex+maltose	Asn	0.15 M	440	1000	1900	2600	1000	91	1	20-28h	dex	88	2	4	Bp	17

Mixed Cluster (See List of Abbreviations, Table 2 (A - K), and Figure 1)

BF Cluster Missing Data: Ind conc, CoUrea2nd = 9; NHase, AMDase, Incg = 999 ; Indl2hppm, Indl8hppm, Indl844hppm, Ind44hppm, IndRate = 9999

C30 Cluster

Table 3.2 (A – K) Frequency Distributions of Variables/Parameters for Experiments Conducted

3.2 A. Vessel

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	BF5L	37	63.8	63.8	63.8
	C30	21	36.2	36.2	100.0
	Total	58	100.0	100.0	

3.2 B. Strain

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	96253	48	82.8	82.8	82.8
	96622	10	17.2	17.2	100.0
	Total	58	100.0	100.0	

3.2 C. Nitrogen

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	PP3	11	19.0	19.0	19.0
	Proflo®	2	3.4	3.4	22.4
	MD	17	29.3	29.3	51.7
	HyC	27	46.6	46.6	98.3
	Pharmamedia	1	1.7	1.7	100.0
	Total	58	100.0	100.0	

3.2 D. Carbohydrate

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	dextrose+maltose	42	72.4	72.4	72.4
	maltoseR3A	9	15.5	15.5	87.9
	dextrose+MG	2	3.4	3.4	91.4
	dextrose+GL	3	5.2	5.2	96.6
	GL	2	3.4	3.4	100.0
	Total	58	100.0	100.0	

Table 3.2 Frequencies (*cont.*)

3.2 E. Inducer

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	none	5	8.6	8.6	8.6
	AC / AN	26	44.8	44.8	53.4
	Asn	27	46.6	46.6	100.0
	Total	58	100.0	100.0	

3.2 F. Inducer Concentration

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	300 ppm	2	3.4	3.8	3.8
	350 ppm	10	17.2	18.9	22.6
	500 ppm	14	24.1	26.4	49.1
	0.1 M	4	6.9	7.5	56.6
	0.15 M	21	36.2	39.6	96.2
	0.2 M	2	3.4	3.8	100.0
	Total	53	91.4	100.0	
Missing	9	5	8.6		
Total		58	100.0		

Table 3.2 Frequencies (*cont.*)

3.2 G. Inducer Rate (µl / min)

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	50	1	1.7	1.9	1.9
	80	4	6.9	7.5	9.4
	120	15	25.9	28.3	37.7
	126	1	1.7	1.9	39.6
	150	1	1.7	1.9	41.5
	350	1	1.7	1.9	43.4
	400	1	1.7	1.9	45.3
	450	1	1.7	1.9	47.2
	476	3	5.2	5.7	52.8
	500	1	1.7	1.9	54.7
	625	1	1.7	1.9	56.6
	666	1	1.7	1.9	58.5
	750	1	1.7	1.9	60.4
	870	1	1.7	1.9	62.3
	1000	9	15.5	17.0	79.2
	2500	10	17.2	18.9	98.1
	3300	1	1.7	1.9	100.0
	Total	53	91.4	100.0	
Missing	9999	5	8.6		
Total		58	100.0		

3.2H. Cobalt / Urea 2nd Addition

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	4-6h	16	27.6	28.1	28.1
	20-28h	38	65.5	66.7	94.7
	>28h	3	5.2	5.3	100.0
	Total	57	98.3	100.0	
Missing	9	1	1.7		
Total		58	100.0		

Table 3.2 Frequencies (*cont.*)

3.2 I. Dextrose / Maltose / YEMEA

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	none	39	67.2	67.2	67.2
	dextrose	13	22.4	22.4	89.7
	YEMEA	4	6.9	6.9	96.6
	maltose	2	3.4	3.4	100.0
	Total	58	100.0	100.0	

3.2 J. Inoculum State

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	plate	27	46.6	46.6	46.6
	Fr	10	17.2	17.2	63.8
	Bp	21	36.2	36.2	100.0
	Total	58	100.0	100.0	

Table 3.2 Frequencies (*cont.*)

3.2 K. Inoculum Mass (g)

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	2	1	1.7	2.2	2.2
	4	1	1.7	2.2	4.3
	5	2	3.4	4.3	8.7
	8	1	1.7	2.2	10.9
	9	1	1.7	2.2	13.0
	11	1	1.7	2.2	15.2
	14	1	1.7	2.2	17.4
	15	4	6.9	8.7	26.1
	16	2	3.4	4.3	30.4
	17	2	3.4	4.3	34.8
	18	2	3.4	4.3	39.1
	19	3	5.2	6.5	45.7
	20	3	5.2	6.5	52.2
	22	2	3.4	4.3	56.5
	27	1	1.7	2.2	58.7
	28	1	1.7	2.2	60.9
	30	12	20.7	26.1	87.0
	32	2	3.4	4.3	91.3
	33	1	1.7	2.2	93.5
	66	2	3.4	4.3	97.8
	140	1	1.7	2.2	100.0
	Total	46	79.3	100.0	
Missing	999	12	20.7		
Total		58	100.0		

Frequency = sample number of variable.
Percent = variable % of total sample number.
Valid Percent = Percent excluding missing data.
Cumulative Percent = Valid Percent totaled.

Figure 3.1 Hierarchical Cluster Analysis

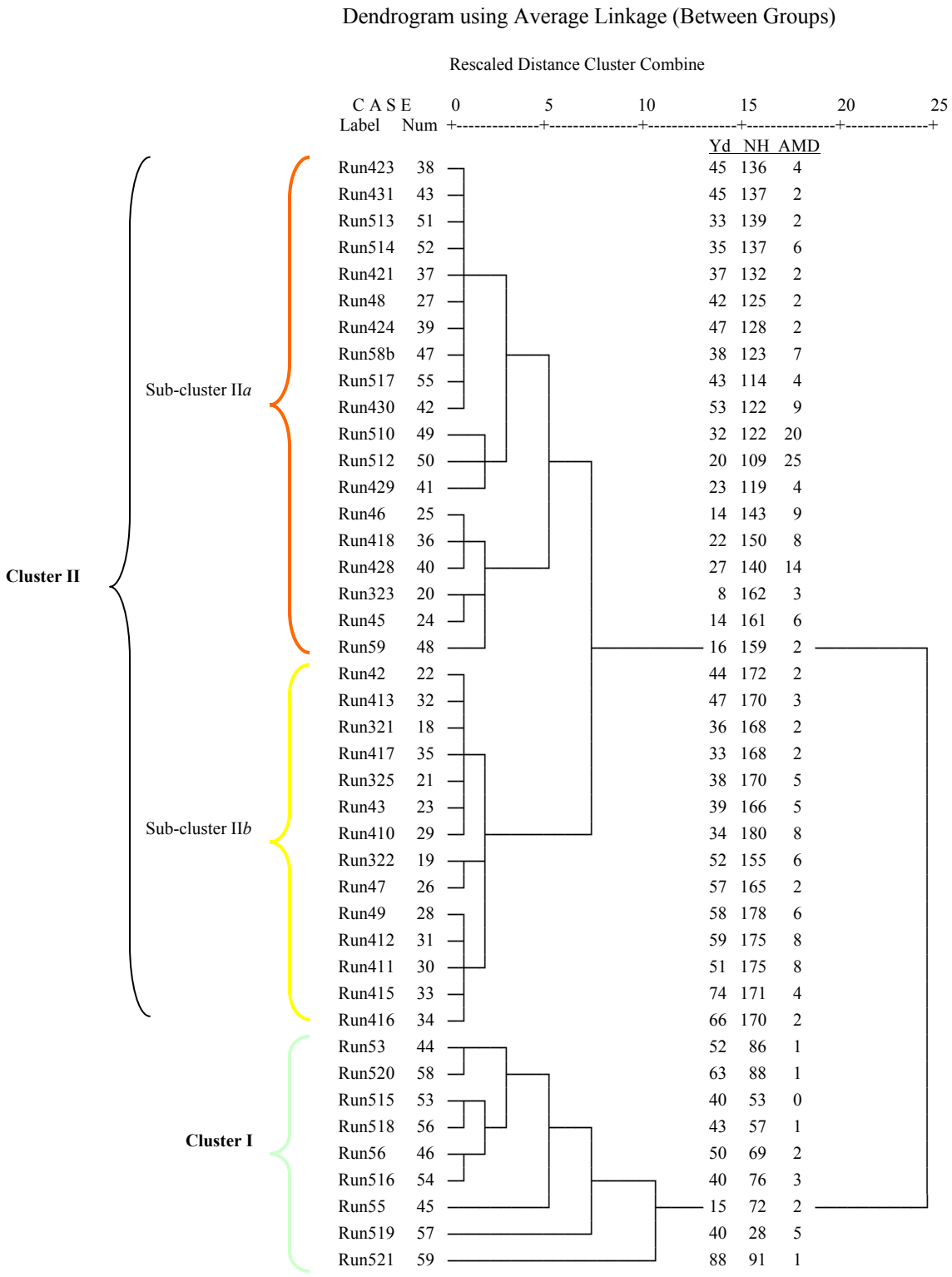
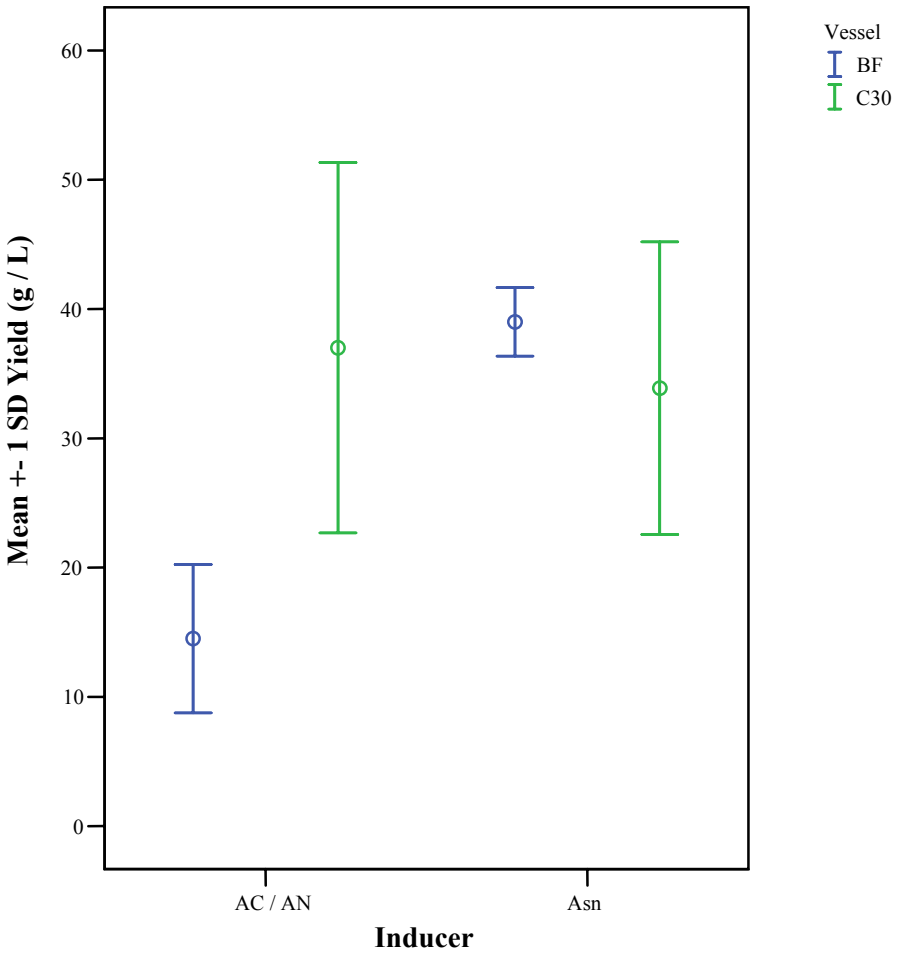


Figure 3.2 Mixed Cluster Biomass Yield vs. Inducer vs. Vessel



AC / AN : BF, n = 4; AC / AN : C30, n = 4; Asn : BF, n = 3; Asn : C30, n = 8

BF Mixed Cluster

The BF Mixed Cluster comprises those runs within Sub-cluster II*a* that were performed in the BF. These runs were characterized as having low biomass yields, high NHase activities, and high or low AMDase activities. Data frequencies revealed that 75 % were dosed with a total of 500 ppm AC / AN, and 75 % employed Hy-Cotton 7803® (HyC) as additional nitrogen source, and 25 % of the runs had extra dextrose added. For runs with 500 ppm AC / AN as the inducer, biomass yield had highly significant correlation coefficients of $^{-}1.00$ at the 0.01 confidence level with the AC / AN inducer concentration at 12 h (Ind 12 h ppm) and 18 h (Ind 18 h ppm). When the analysis of biomass yield was expanded to all runs in the BF with 500 ppm AC / AN as inducer, which included runs without NHase or AMDase data, there was a highly significant positive $^{+}0.845$ correlation at the 0.01 confidence level between biomass yield and inoculum mass (Incg). A graph of this data clearly shows two yield groups: a) lower biomass yields correlated with ≤ 20 g cell wet weight (cww) inoculum, and b) greater biomass yields correlated with inoculum levels ≥ 30 g (cww) (Figure 3.3). When the initial step inducer addition was delayed to 18 h, a cell mass yield resulted that was similar to those from runs with lower AC / AN addition rates that began at 0 h.

Although biomass yields were low, the NHase activities were high and variable. As the AC / AN inducer rate (IndR) increased from 50 to 120 µl / min, NHase activity increased (Figure 3.4). In addition, for runs with inducer rates of 120 µl / min, as the inducer concentration (IndC) increased from 350 to 500 ppm AC / AN, the NHase activity increased. For plate-derived inocula with masses 30 – 32 g, higher NHase

activity was induced when HyC was the nitrogen source in comparison to Proflo® (Figure 3.5).

AMDase activity was affected by the same variables that affected NHase, however not in the same manner. As the AC / AN inducer rate increased from 50 to 120 µl / min, AMDase activity decreased; and for runs with AC / AN inducer rates of 120 µl / min, as the AC / AN concentration increased, AMDase decreased. For 30 – 32 g plate-derived inocula, lower AMDase activity was induced when HyC was used as the nitrogen source over Proflo®. Within the plate-derived inoculum runs, lower AMDase activity was induced with DAP 96253.

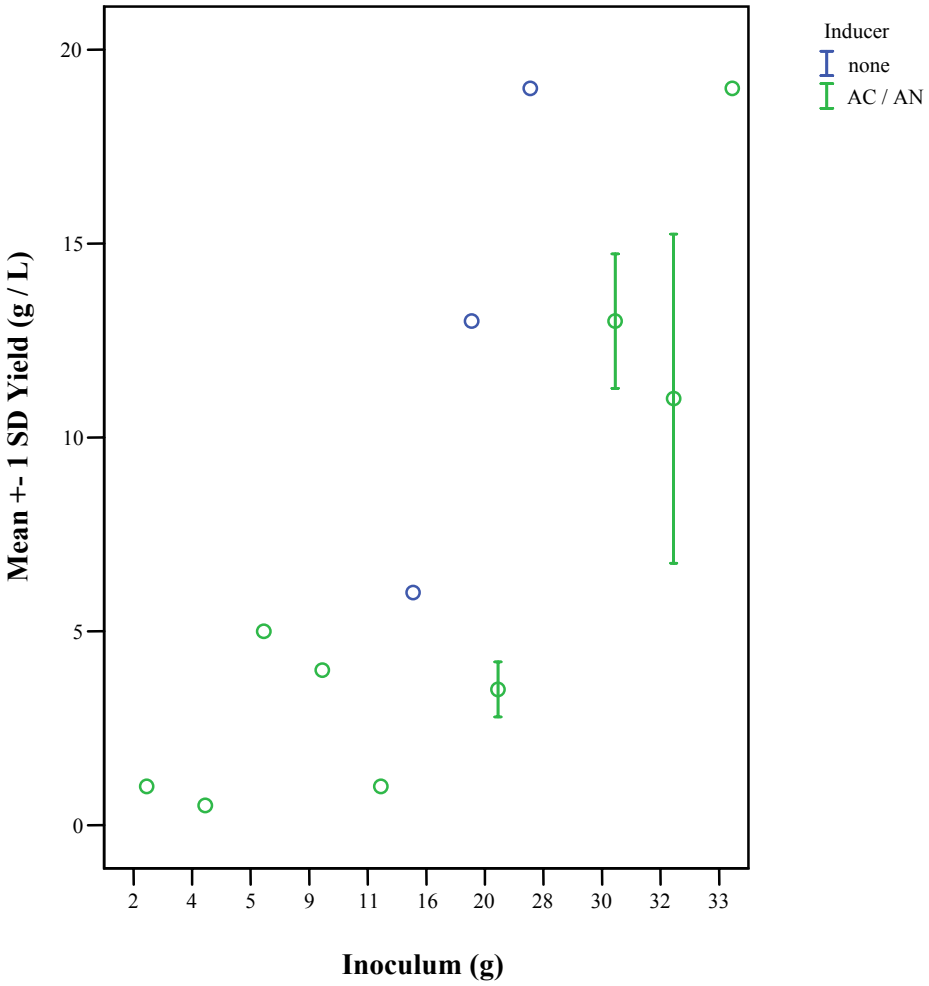
Runs 48, 421, and 58b (see Table 3.1 and Figure 3.1) were all run in the BF and were all asparagine (Asn) induced. Run 48 with an inoculum mass of 30 g and Asn inducer rate of 2500 µl / min produced higher biomass than Runs 421 and 58b with inoculum masses of 8 and 5 g, respectively, and Asn inducer rates of 120 and 126 µl / min, respectively (Figure 3.6). Figure 3.7 also showed highly significant positive correlation between increased yield and increased Asn inducer rate, with the highest biomass yield associated with extra dextrose addition. The positive relationship between increased yield, and increased inoculum mass and extra carbohydrate was conserved when the data was expanded to all Asn induced runs performed in the BF (Figure 3.8).

NHase activities within this cluster were also affected by carbohydrate as well as other parameters. There was a highly significant positive correlation between NHase activities and free dextrose concentrations, and a highly significant negative correlation between NHase and 0 – 44 h Asn concentration. Of the two runs, 421 and 58b, with low

inoculum mass, low inducer rate, and equivalent Asn inducer concentration, Run 421 [with DAP 96622, lower maltose addition, and HyC as the nitrogen source] had increased NHase activity over Run 58b [with DAP 96253, higher maltose addition as maltoseR3A and MD® as the replacement nitrogen source].

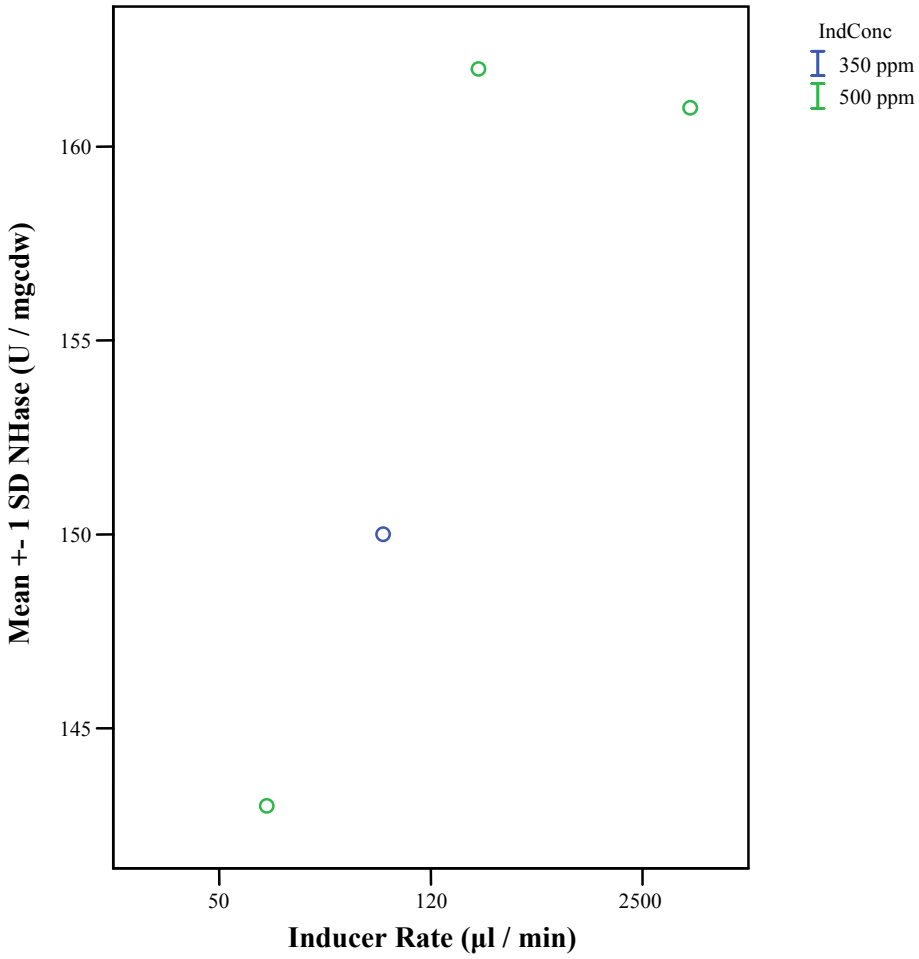
Strain variability was found with AMDase activity as well as with NHase activity (Figure 3.9). DAP 96253 [with higher maltose and Cottonseed Hydrolysate or Cottonseed Hydrolysate-Ultrafiltered [MD], (Marcor Devolpment Corp.) as the added nitrogen source] induced higher AMDase activity than DAP 96622 [with R3A and either MD or HyC as the replacement nitrogen source]. Furthermore, as Asn concentration increased between 18 and 44 h with MD as the replacement nitrogen source, DAP 96253 induced greater AMDase activity than DAP 96622 (Figure 3.10).

Figure 3.3 All BF AC / AN 500 ppm Induced Biomass Yield vs. Inoculum Mass vs. Inducer



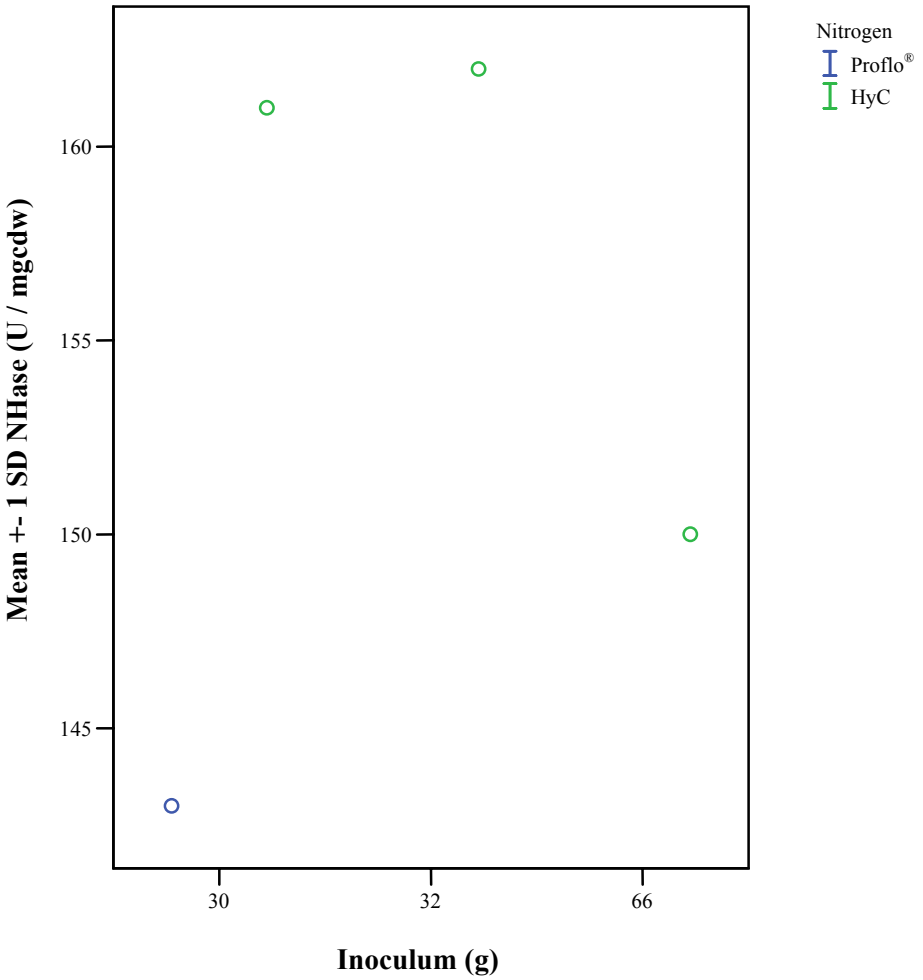
Discrete circles, n = 1; 20 g : AC / AN, n = 2; 30 g : AC / AN, n = 3; 32 g : AC / AN, n = 2

Figure 3.4 BF Mixed Cluster AC / AN Induced NHase vs. Inducer Rate vs. Inducer Concentration



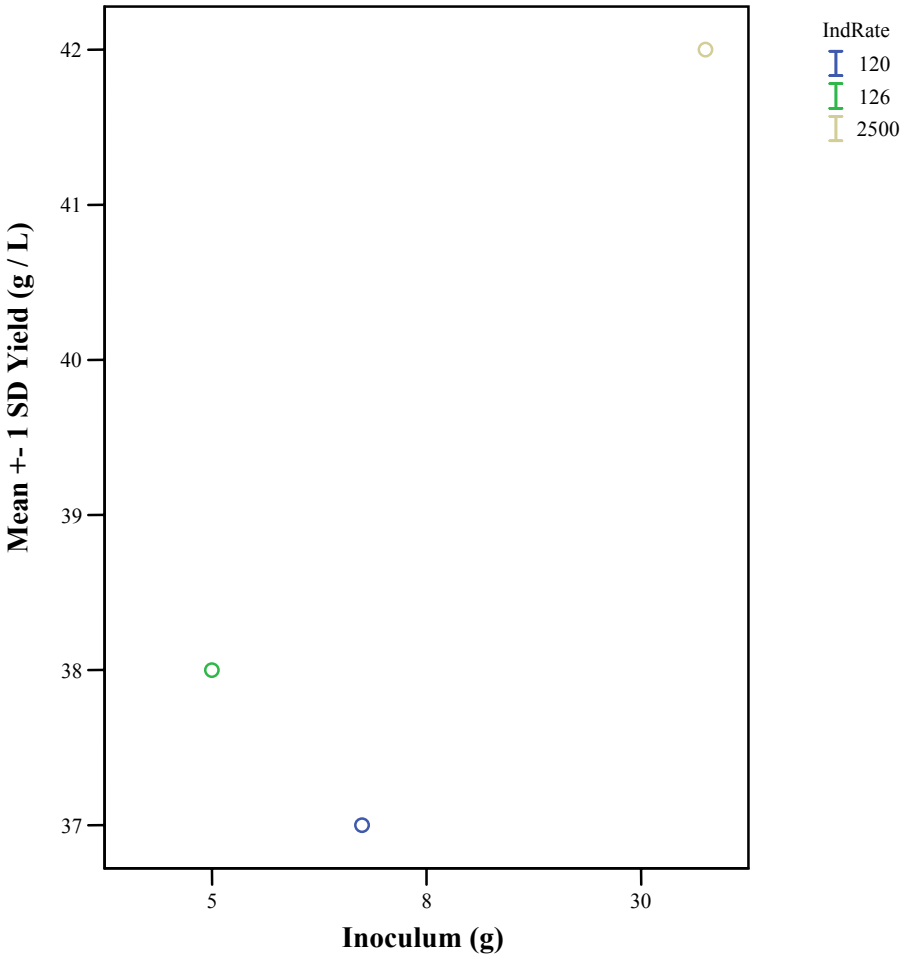
Discrete circles, n = 1

Figure 3.5 BF Mixed Cluster AC / AN Induced NHase vs. Inoculum Mass vs. Nitrogen



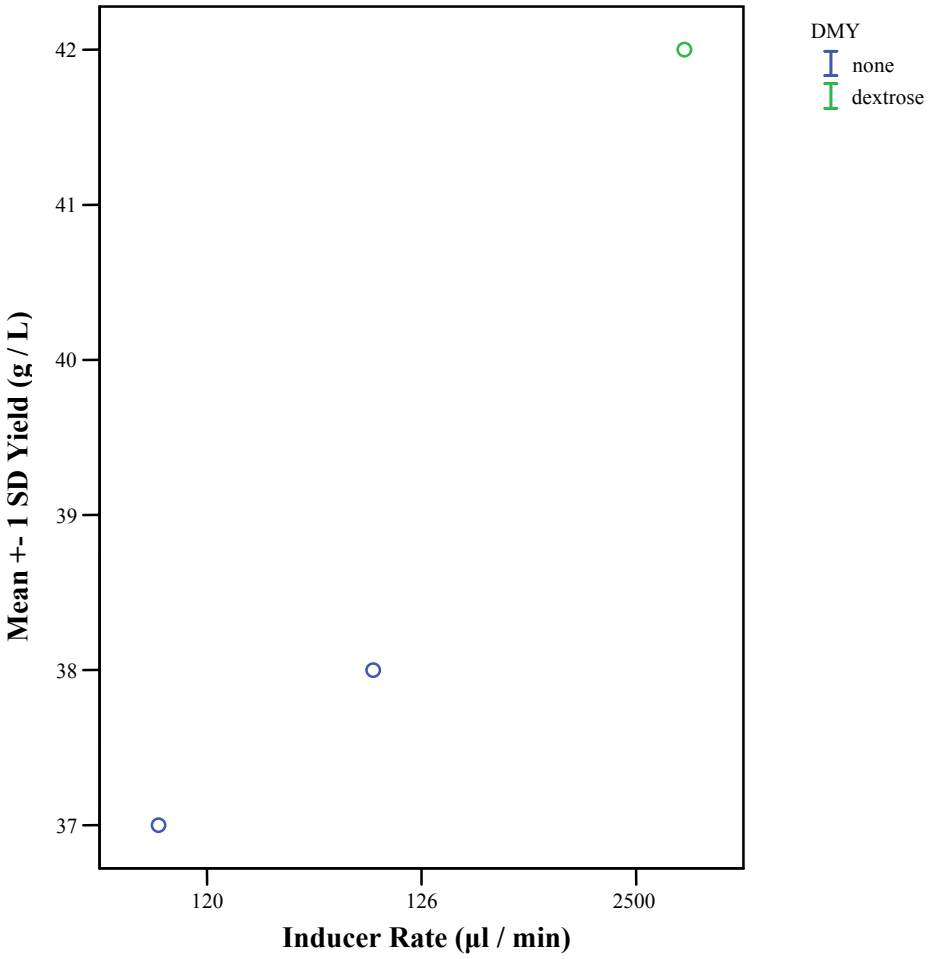
Discrete circles, n = 1

Figure 3.6 BF Mixed Cluster Asn Induced Yield vs. Inoculum Mass vs. Inducer Rate (µl / min)



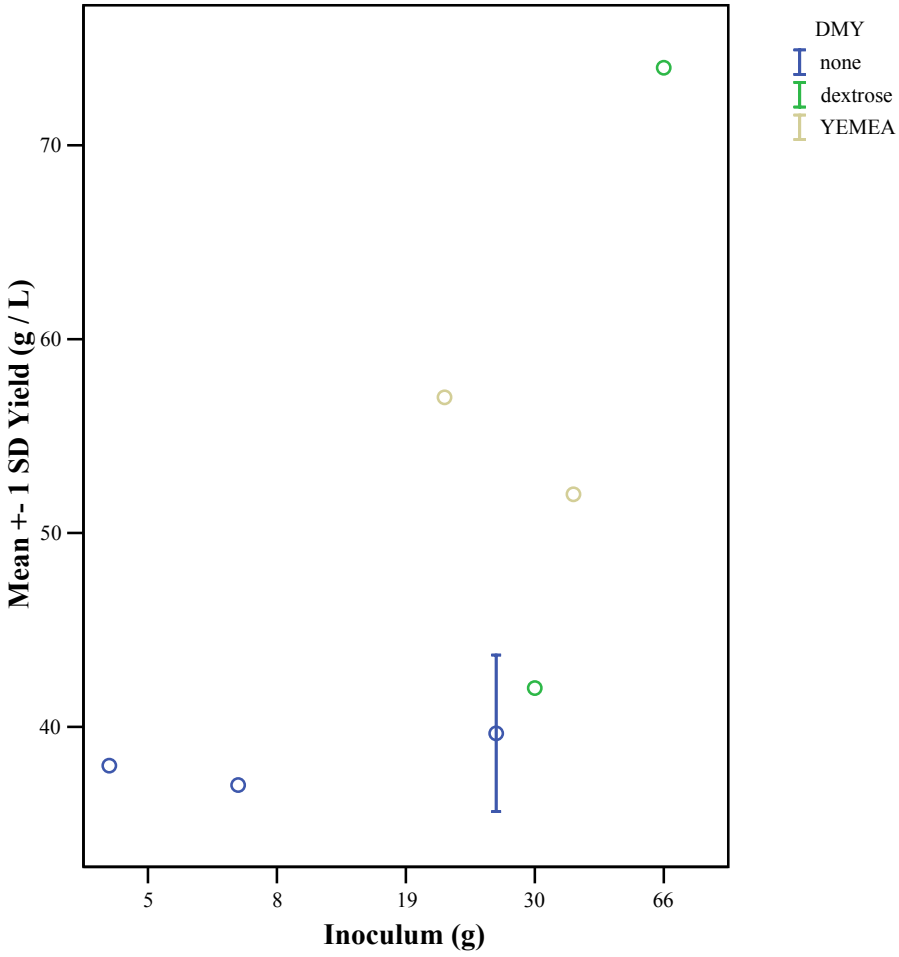
Discrete circles, n = 1

Figure 3.7 BF Mixed Cluster Asn Induced Biomass Yield vs. Inducer Rate vs. Supplemental Carbohydrate (DMY)



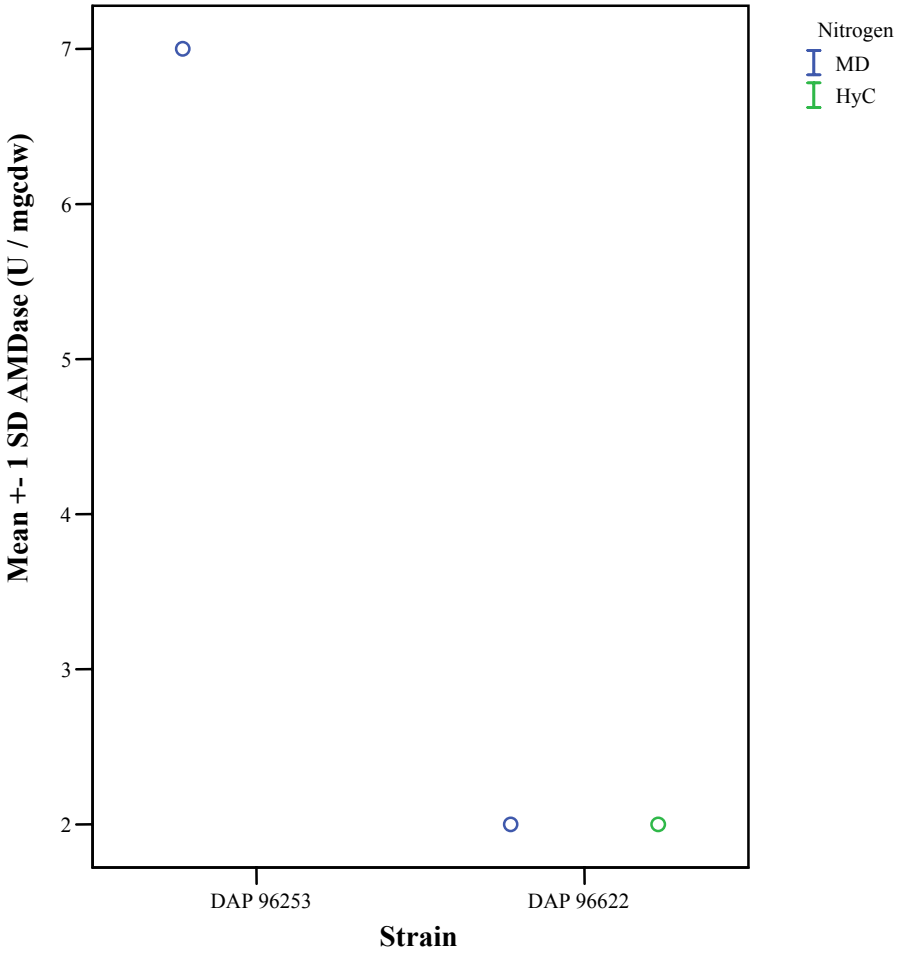
Discrete circles, n = 1

Figure 3.8 All BF Asn Induced Biomass Yield vs. Inoculum Mass vs. Supplemental Carbohydrate (DMY)



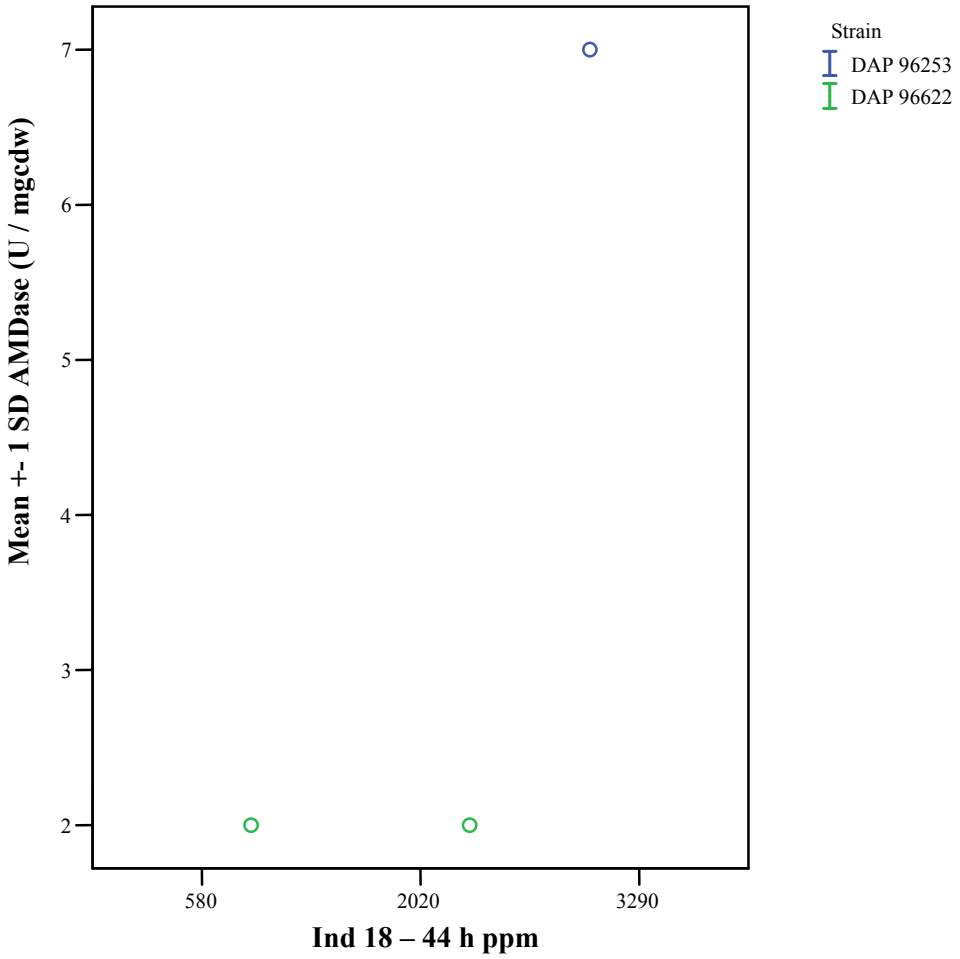
Discrete circles, n = 1; 30 g : none, n = 4

Figure 3.9 Mixed Cluster BF Asn Induced AMDase vs. Strain vs. Nitrogen



Discrete circles, n = 1

Figure 3.10 Mixed Cluster BF Asn Induced AMDase vs. Asn Concentration t = 18 – 44 h vs. Strain



Discrete circles, n = 1

C30 Mixed Cluster

All of the runs within Sub-cluster II*a* that were performed in the C30 (C30 Mixed Cluster) were initiated with inocula derived from biphasic flask cultures (Table 3.1). Where AC / AN was employed as inducer, those runs with dextrose + the dextrose equivalent Malta Gran® 18M [MG] (Primera Foods, Cameron, WI) as the major carbohydrates obtained significantly lower biomass than with dextrose + the low dextrose equivalent corn syrup solid Cargill Dry GL™ 01925 [GL] (Cargill, Inc., Cedar Rapids, IA) (Figure 3.11). Runs 430 and 431 (dextrose + GL) received ~ 40 ppm less AC / AN over the t = 18 – 44 h time period than Runs 428 and 429 (dextrose + MG). When runs with initial AC / AN additions at 8 h are considered, biomass yield decreased as inducer rate increased (Figure 3.12). However, for runs with initial AC / AN additions at 23 – 25 h, higher biomass yield was produced when the start time was at 25 h with higher inducer rate than at 23 h with lower (high) inducer rate.

The relationship between NHase activity, initial AC / AN addition time, and inducer rate differed from that seen with cell mass. When runs with initial AC / AN additions at 8 h are considered, NHase activity increased as the inducer rate increased. Similarly, when initial AC / AN additions were delayed to 23 – 25 h, NHase activity increased as the inducer rate increased (Figure 3.13).

The effect on AMDase activity by the AC / AN addition rate and the initial AC / AN addition time differed from both NHase activity and cell mass. AMDase activity was found to decrease with increasing AC / AN addition when runs with 8 h initial AC / AN

additions were compared (Figure 3.14). However, when the AC / AN addition start time was delayed to 23 or 25 h, AMDase activity increased as the inducer rate increased.

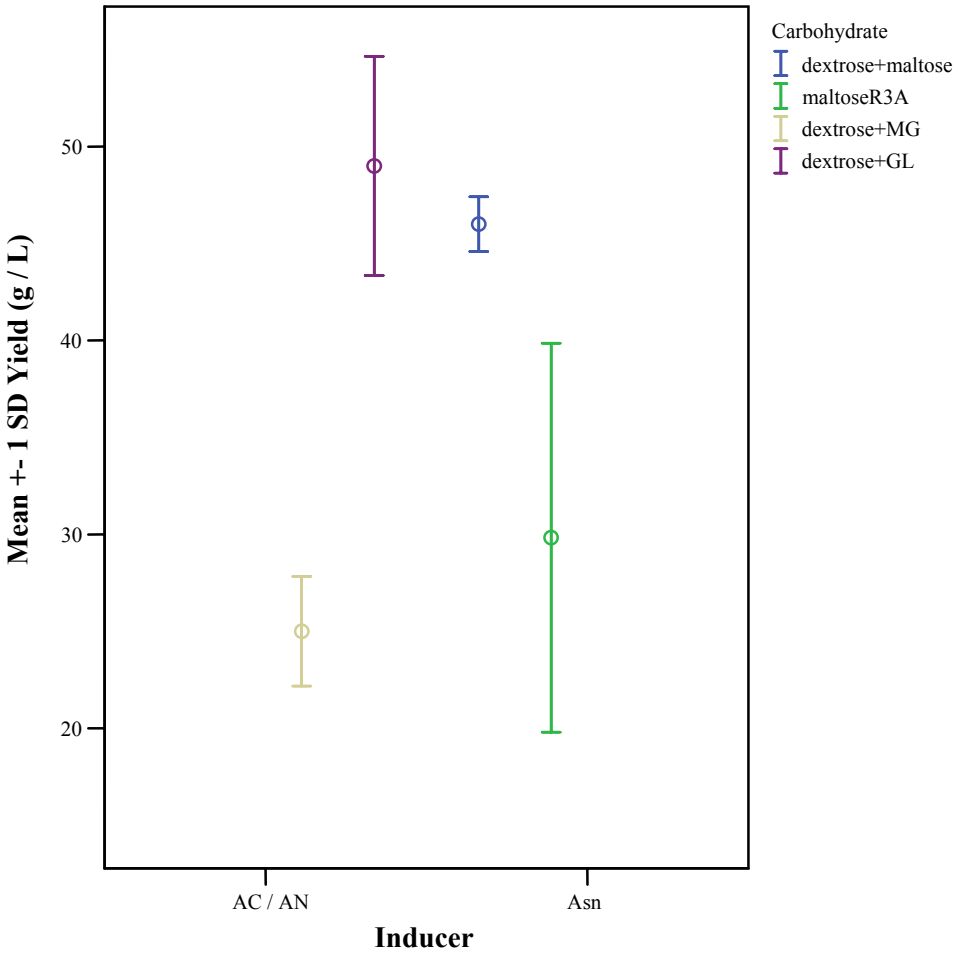
Asn induced runs that comprised the C30 Mixed Cluster also showed an effect on cell mass by the inducer addition rate. Figure 3.15 shows that, for runs with the same inoculum mass, as the Asn addition rate increased, biomass yield decreased. When runs with the same inducer rate are considered, as the inoculum mass increased, yield increased. Highly significant positive correlations were found between biomass yield and Asn concentration at 0 – 12 h and 0 – 18 h, and 0 – 24 h free dextrose concentration.

NHase activity was also affected by the Asn addition rate and the inoculum mass. Figure 3.16 shows that, for runs with the same inoculum mass, NHase activity decreased as the Asn addition rate increased. In addition, for runs with the same Asn addition rate as Incg increased, NHase decreased.

Effects of Asn addition rates on AMDase activities were as follows: at inoculum mass = 15 g, as the inducer rate increased from 400 or 500 µl / min to 870 µl / min, AMDase activity increased (Figure 3.17). As the inoculum mass increased within the same inducer rate (1000 µl / min), AMDase decreased. Significant (at the 0.05 confidence level) negative correlations were found between AMDase activity and biomass yield, Asn concentration from 0 – 12 h and 0 – 18 h. Effects on AMDase activities in the mixed cluster Asn induced C30 runs were also found when runs were considered that included strain DAP 96253, nitrogen as HyC, and carbohydrate as maltoseR3A. There were highly significant positive correlations between AMDase activity and Asn addition rate as well as between Asn addition rate and 18 – 44 h and 0 –

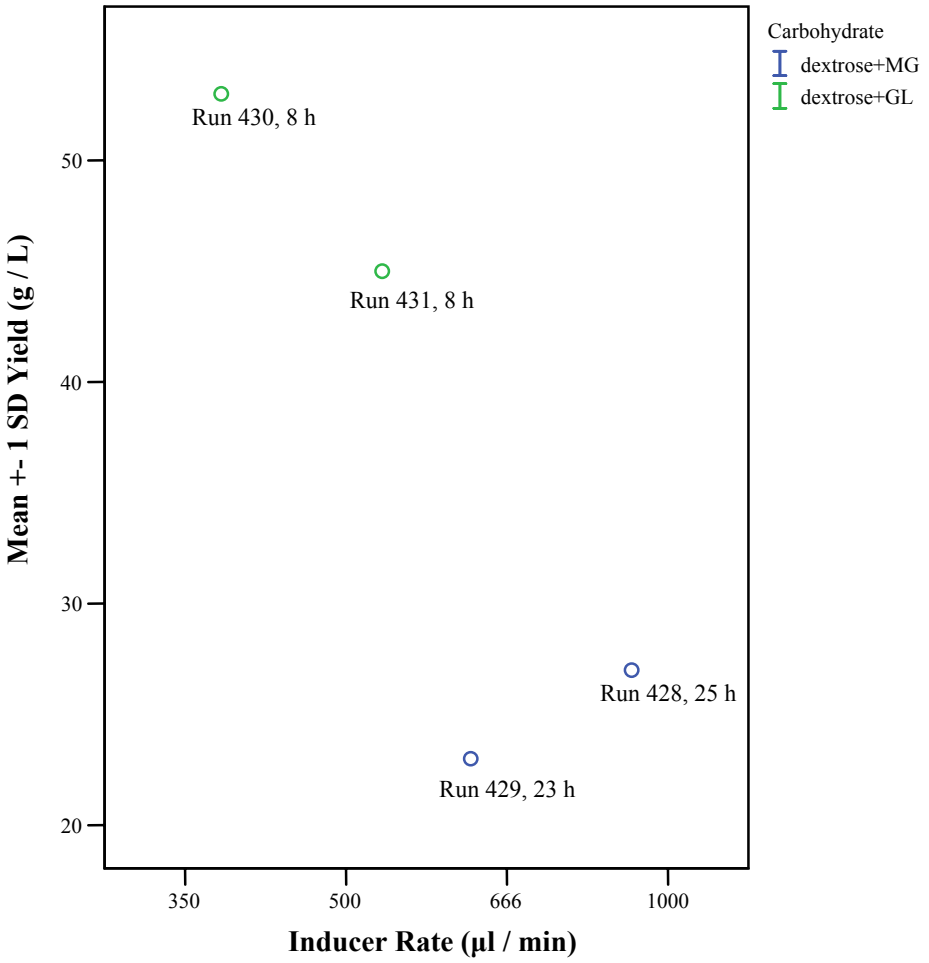
44 h Asn concentrations (Table 3.3). There was also highly significant negative correlation between AMDase and NHase activities.

Figure 3.11 Mixed Cluster C30 Yield vs. Inducer vs. Carbohydrate



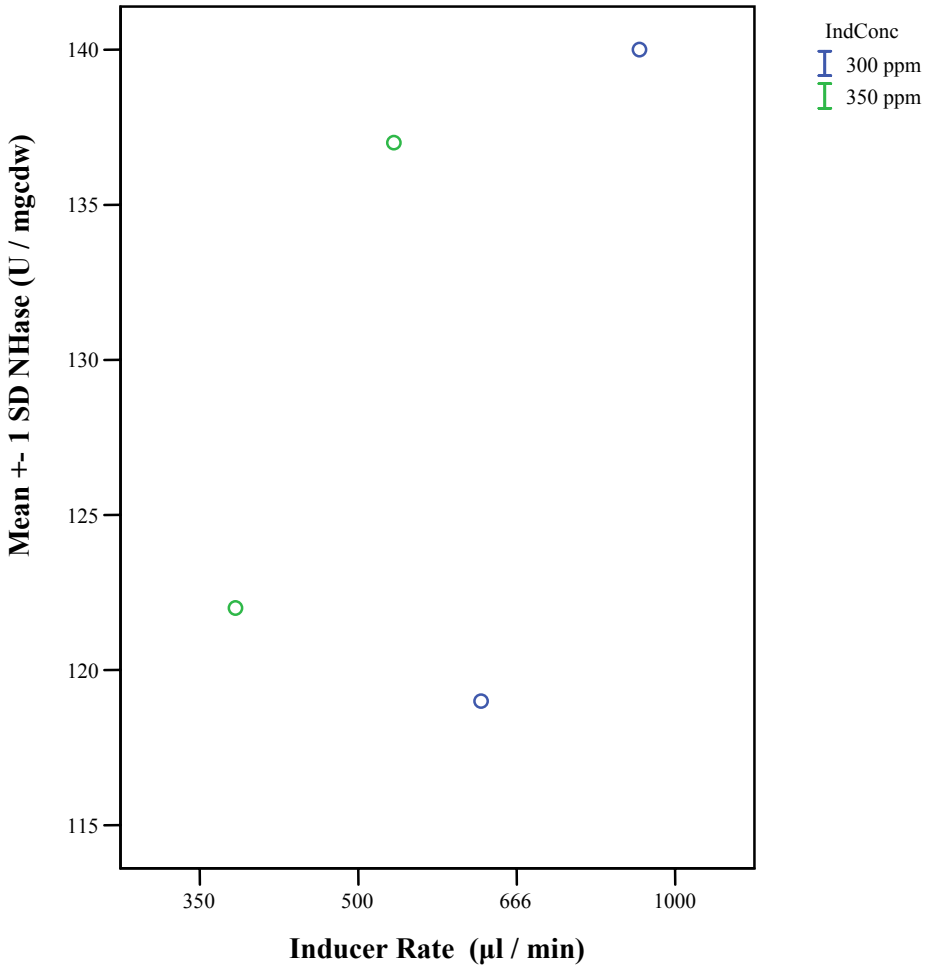
AC / AN : dextrose+MG, n = 2; AC / AN : dextrose+GL, n = 2;
Asn : dextrose+maltose, n = 3; Asn : maltoseR3A, n = 6

Figure 3.12 Mixed Cluster C30 AC / AN Induced Yield vs. Inducer Rate vs. Carbohydrate



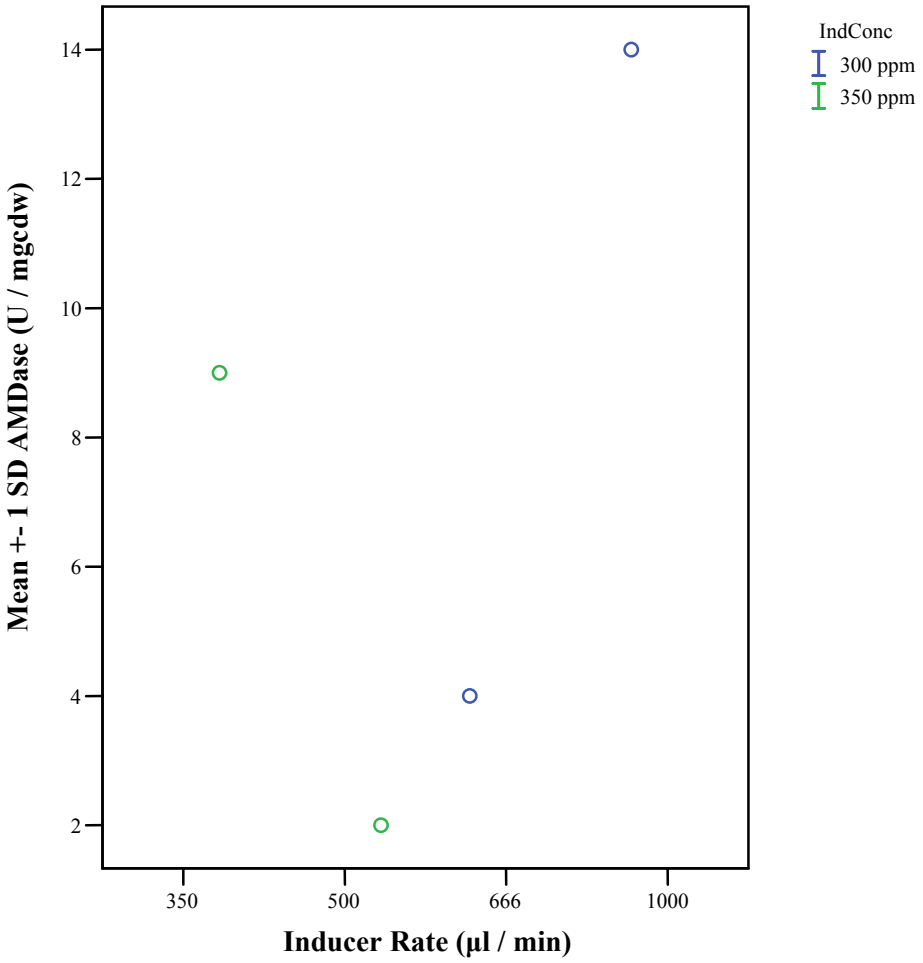
Data points are labeled with Run code and initial AC / AN addition times (h).
Discrete circles, n = 1

Figure 3.13 Mixed Cluster C30 AC / AN Induced NHase vs. Inducer Rate vs. Inducer Concentration



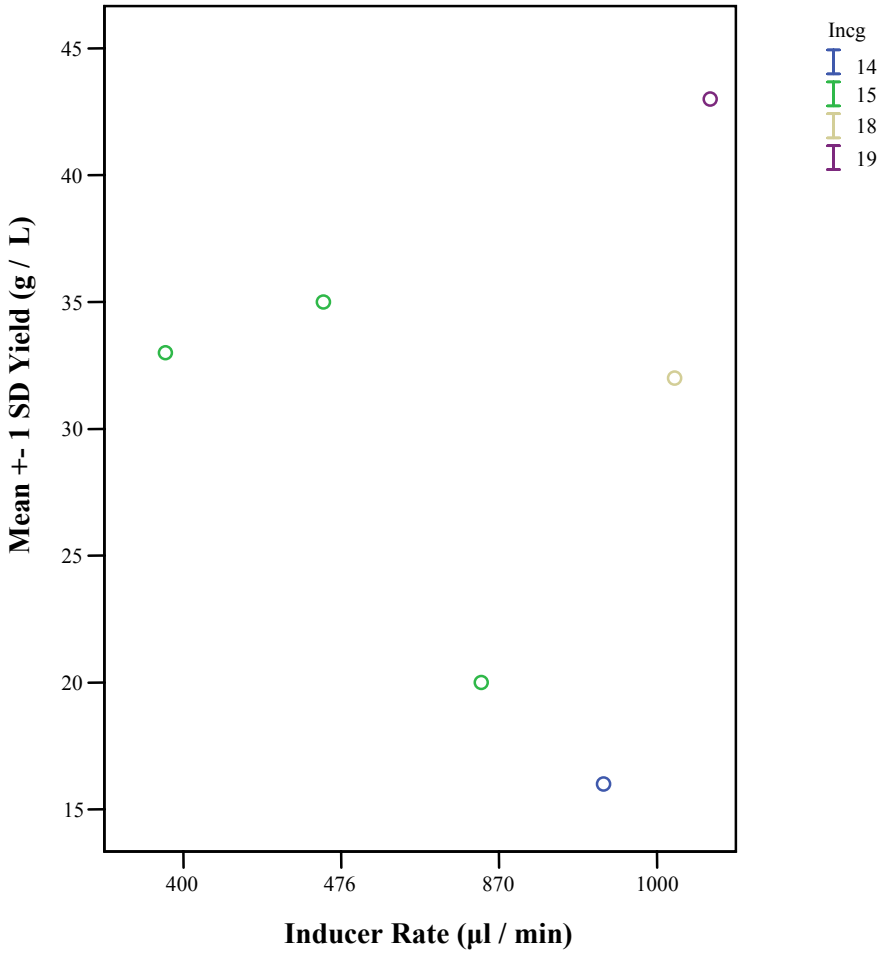
Discrete circles, n = 1

Figure 3.14 Mixed Cluster C30 AC / AN Induced AMDase vs. AC / AN Addition Rate vs. AC / AN Concentration



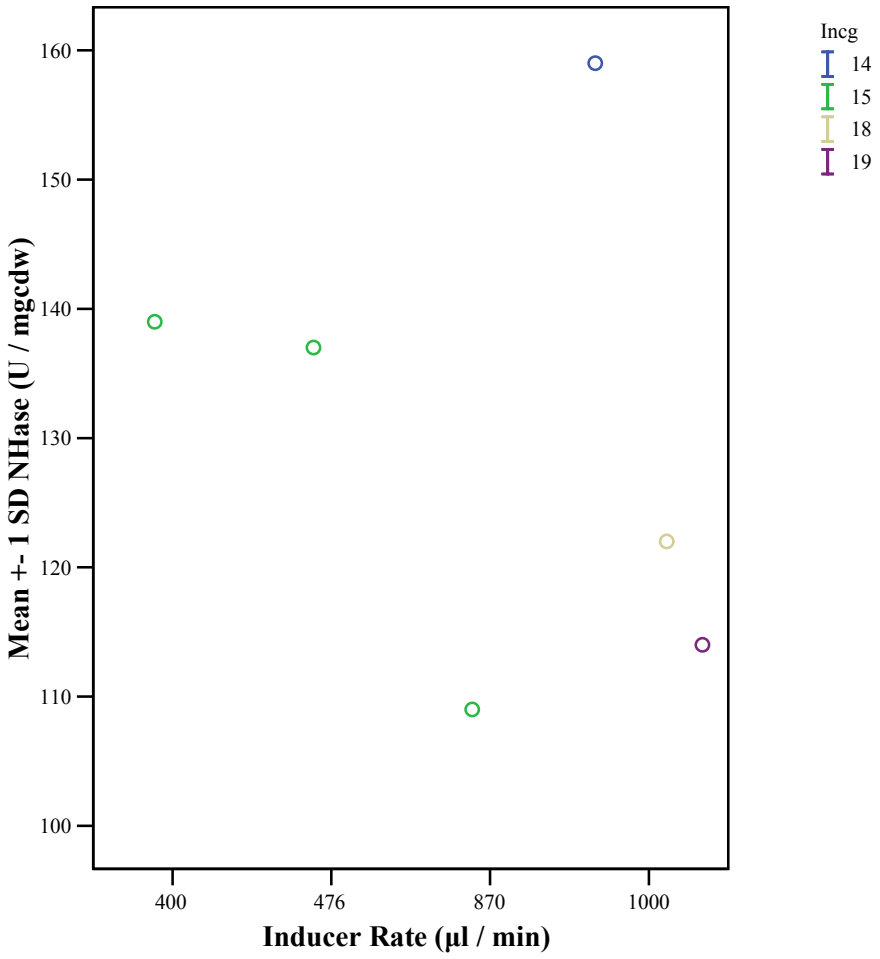
Discrete circles, n = 1

Figure 3.15 Mixed Cluster C30 Asn Induced Biomass Yield vs. Asn Addition Rate vs. Inoculum Mass (g)



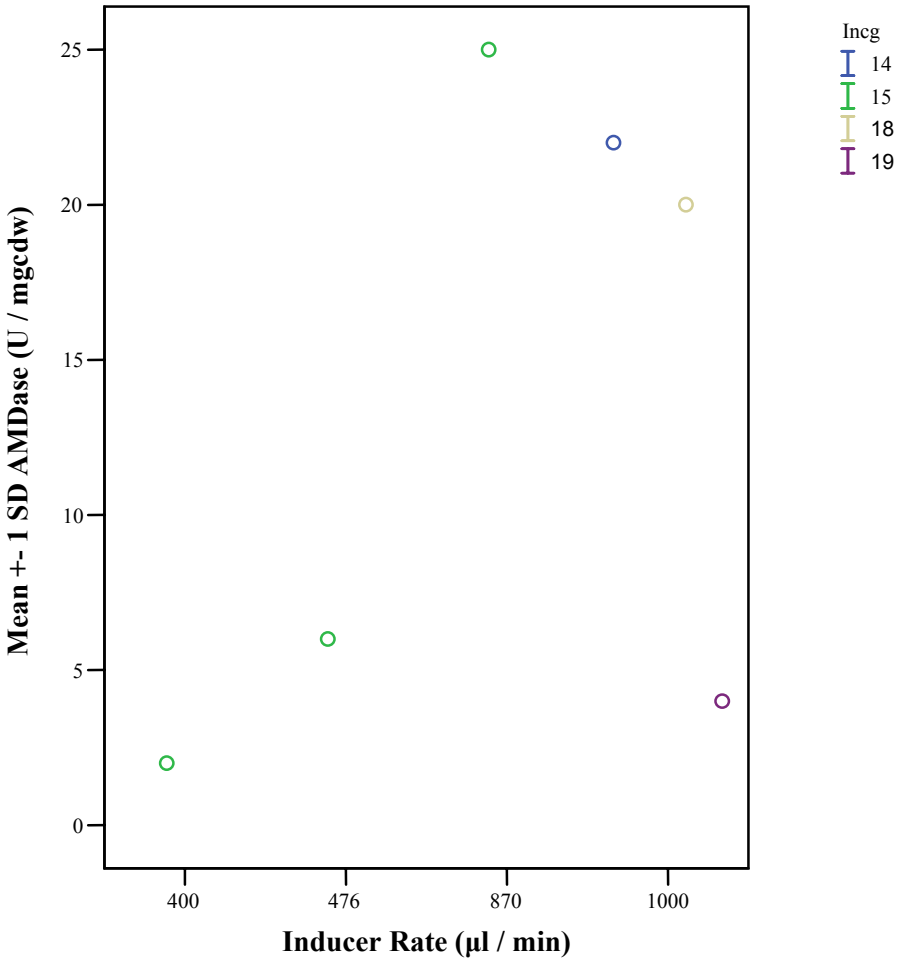
Discrete circles, n = 1

Figure 3.16 Mixed Cluster C30 Asn Induced NHase vs. Asn Addition Rate vs. Inoculum Mass (g)



Discrete circles, n = 1

Figure 3.17 Mixed Cluster C30 Asn Induced AMDase vs. Asn Addition Rate vs. Inoculum Mass (g)



Discrete circles, n = 1

Table 3.3 Correlations C30 Mixed Cluster Asn Induced MaltoseR3A Hy-Cotton 7803® DAP 96253

			Yield	NHase	AMDase	Incg	IndRate	Ind12hppm	Ind18hppm	In1844ppm	Ind44hppm	dex0to24	dex24to44
Spearman's rho	Yield	Correlation Coefficient	1.000	.500	-.500	.	-.500	1.000	1.000	-1.000	-1.000	1.000	.
		Sig. (2-tailed)	.	.667	.667	.	.667	.	.	1.000	1.000	.	.
		N	3	3	3	3	3	2	2	2	2	2	2
	NHase	Correlation Coefficient	.500	1.000	-1.000**	.	-1.000**	1.000	1.000	-1.000	-1.000	1.000	.
		Sig. (2-tailed)	.667	.	.000	.	.000	.	.	1.000	1.000	.	.
		N	3	3	3	3	3	2	2	2	2	2	2
	AMDase	Correlation Coefficient	-.500	-1.000**	1.000	.	1.000**	-1.000	-1.000	1.000	1.000	-1.000	.
		Sig. (2-tailed)	.667	.000	.	.	.	1.000	1.000	.	.	1.000	.
		N	3	3	3	3	3	2	2	2	2	2	2
	Incg	Correlation Coefficient	.	.	.	.	.	.	.	.	.	.	.
		Sig. (2-tailed)	.	.	.	.	.	.	.	.	.	.	.
		N	3	3	3	3	3	2	2	2	2	2	2
	IndRate	Correlation Coefficient	-.500	-1.000**	1.000**	.	1.000	-1.000	-1.000	1.000	1.000	-1.000	.
		Sig. (2-tailed)	.667	.000	.	.	.	1.000	1.000	.	.	1.000	.
		N	3	3	3	3	3	2	2	2	2	2	2
	Ind12hppm	Correlation Coefficient	1.000**	1.000**	-1.000	.	-1.000	1.000	1.000	-1.000	-1.000	.	.
		Sig. (2-tailed)	.	.	1.000	.	1.000	.	.	1.000	1.000	.	.
		N	2	2	2	2	2	2	2	2	2	1	1
	Ind18hppm	Correlation Coefficient	1.000**	1.000**	-1.000	.	-1.000	1.000**	1.000	-1.000	-1.000	.	.
		Sig. (2-tailed)	.	.	1.000	.	1.000	.	.	1.000	1.000	.	.
		N	2	2	2	2	2	2	2	2	2	1	1
	In1844ppm	Correlation Coefficient	-1.000	-1.000	1.000**	.	1.000**	-1.000	-1.000	1.000	1.000	.	.
		Sig. (2-tailed)	1.000	1.000	.	.	.	1.000	1.000	.	.	.	.
		N	2	2	2	2	2	2	2	2	2	1	1
	Ind44hppm	Correlation Coefficient	-1.000	-1.000	1.000**	.	1.000**	-1.000	-1.000	1.000**	1.000	.	.
		Sig. (2-tailed)	1.000	1.000	.	.	.	1.000	1.000	.	.	.	.
		N	2	2	2	2	2	2	2	2	2	1	1
	dex0to24	Correlation Coefficient	1.000**	1.000**	-1.000	.	-1.000	.	.	.	.	1.000	.
		Sig. (2-tailed)	.	.	1.000	.	1.000	.	.	.	.	.	.
		N	2	2	2	2	2	1	1	1	1	2	2
	dex24to44	Correlation Coefficient	.	.	.	.	.	.	.	.	.	.	.
		Sig. (2-tailed)	.	.	.	.	.	.	.	.	.	.	.
		N	2	2	2	2	2	1	1	1	1	2	2

**. Correlation is significant at the 0.01 confidence level (2-tailed). See List of Abbreviations for codes.

BF Cluster

The runs that grouped into the BF cluster were characterized as having high yields, high NHase activities, and low to medium AMDase activities with AC / AN or Asn as inducers. The frequency distribution of the main nitrogen source was 57 % HyC and 43 % MD; the major carbohydrates were all dextrose + maltose; 60 % of the runs had increased carbohydrate addition (DMY); 86 % of the inoculum state was plate-derived with inoculum masses at 19 – 30 g and 1 frozen-derived inoculum at 66 g. The frequency distribution for AC / AN induced runs in the BF Cluster included: 86 % were with DAP 96253; sources of nitrogen were 43 % HyC and 57 % MD; all AC / AN concentrations were 350 ppm; all AC / AN addition rates were 120 µl / min; 85 % of the runs had extra carbohydrate added; all inocula were plate-derived and ≥ 22 g (plate derived were 22 – 30 g). There were no apparent correlations between biomass yield and any measured variable within this cluster. Also, there were no significant differences between the different nitrogen sources and biomass yield. However, when all AC / AN induced runs performed in the BF are considered, biomass yields from 350 ppm AC / AN induced runs were significantly higher than 500 ppm AC / AN induced runs both with and without increased carbohydrate addition (Figure 3.18 and Figure 3.19). Run 418, with a frozen 66 g inoculum, was only slightly higher than 500 ppm runs (Figure 3.18). Within runs that had data for yield, NHase, and AMDase; the inoculum masses in 350 ppm AC / AN induced runs were generally lower than for 500 ppm AC / AN induced runs; except for Run 418 (Figure 3.20). The highest yields were those from 350 ppm AC / AN induced

runs with plate-derived inocula that had extra carbohydrate addition (Figure 3.20 and Figure 3.21).

The run with the highest NHase activity also had extra carbohydrate (dextrose) added, along with a 30 g inoculum and MD as the main nitrogen source (Figure 3.22 and Figure 3.23). A graph of NHase activity vs. inoculum state vs. inducer concentration showed the following relative order for NHase activities: 350 ppm AC / AN induced runs with plate-derived inocula > 500 ppm induced with plate-derived inocula and 350 ppm induced with frozen-derived inocula (Figure 3.24). Highly significant positive correlations were found between NHase and AMDase activities, and significant positive correlations were found between NHase activity and inoculum mass for runs with plate-derived inocula. A highly significant positive correlation was also calculated between AMDase activities and inoculum mass (Figure 3.25).

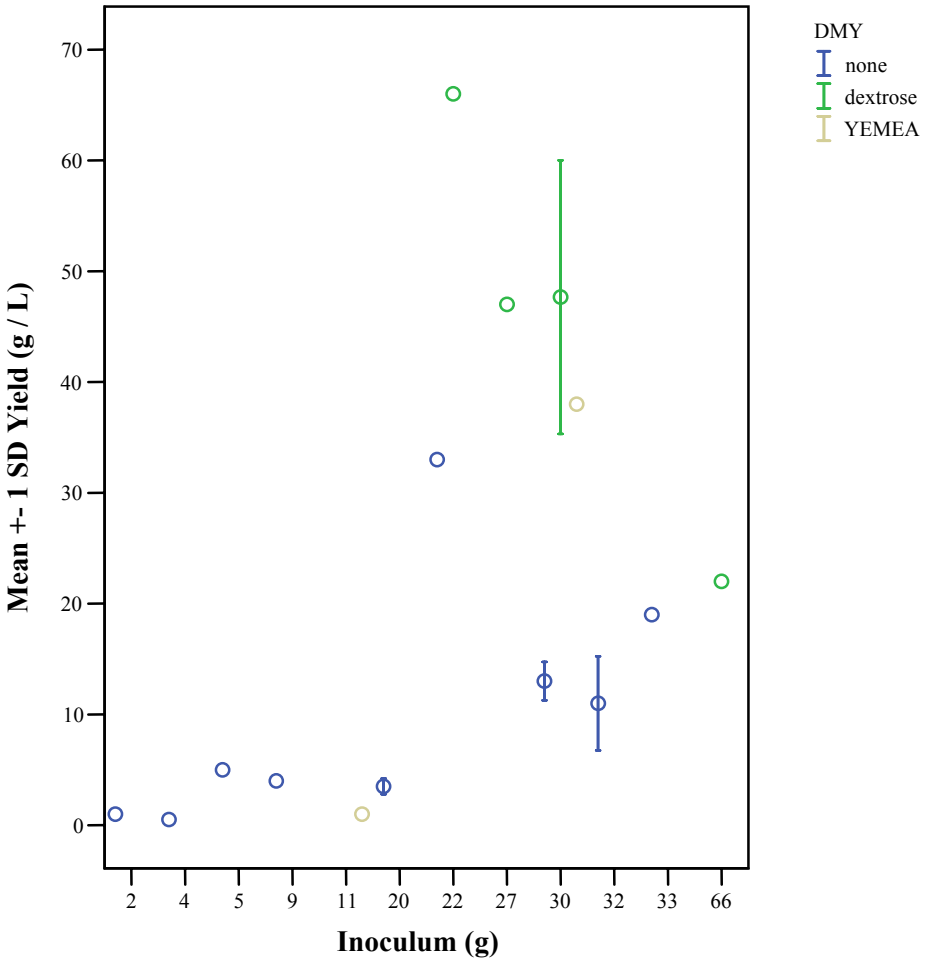
When runs within the BF Cluster that were Asn induced are considered, the highest biomass yields were obtained when there was increased carbohydrate addition (DMY) when compared to runs without extra carbohydrate addition (Figure 3.26). Asn induced runs with high biomass yields had low to high inoculum masses, but those with ≥ 19 g produced the greatest biomass yields, in conjunction with supplemental carbohydrate.

As was the case for AC / AN induced runs within the BF Cluster, all Asn induced NHase activities were high (or very high; 155 – 175 U / mgcdw). The lowest NHase activity for the group was from Run 322 with strain DAP 96253, nitrogen as HyC, and extra carbohydrate as YEMEA. All other runs had DAP 96622 with nitrogen as HyC or MD with no extra carbohydrate or with dextrose (Figure 3.27 and Table 3.1). When all

Asn induced runs performed in the BF (controlled for Run 48 with foam-out) were compared, there was significant positive correlation between increased inoculum mass and increased NHase activity (Figure 3.28).

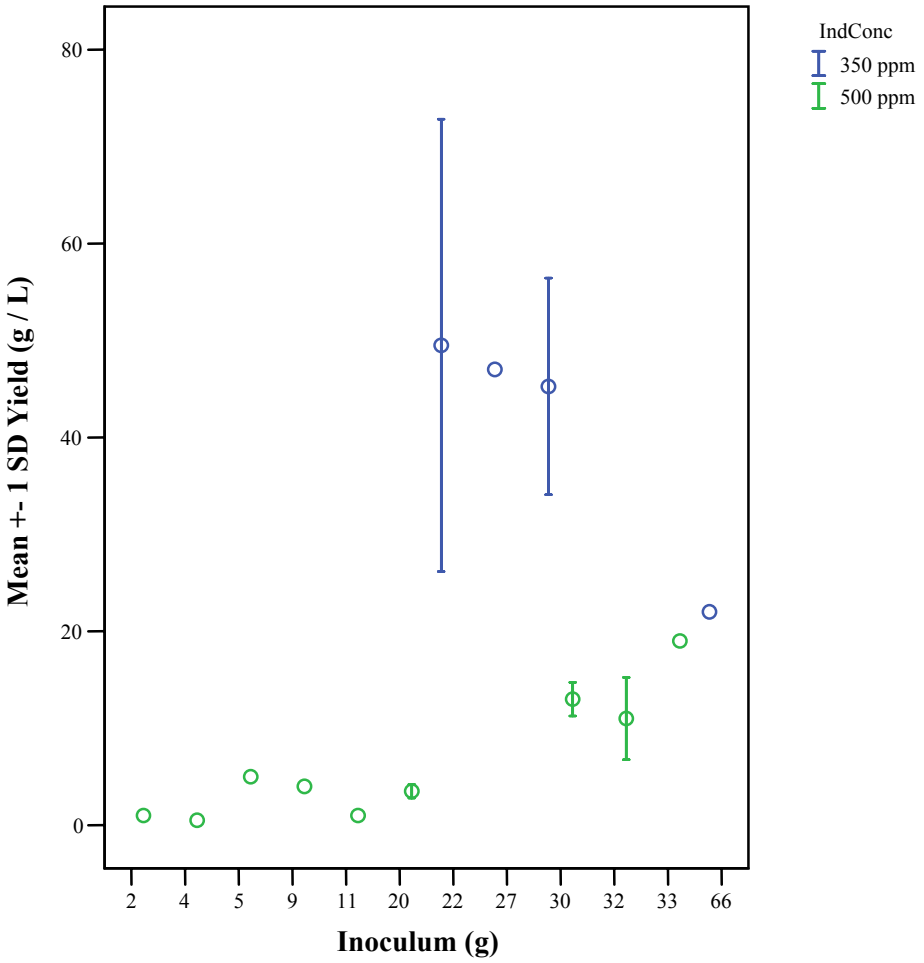
There were no apparent significant correlations, however, with AMDase for this cluster. The minimum AMDase activity = 2, the maximum = 8, and the mean = 4.14 ± 2.3 SD. This is similar to Asn induced mixed cluster BF runs that had minimum AMDase activity = 2, maximum = 7 and the mean = 3.67 ± 2.9 SD. When AMDase activities were compared by strain and nitrogen, there was a difference between strains with nitrogen MD, but not with HyC (Figure 3.29). DAP 96253 with MD induced higher AMDase activity than with HyC, and also higher than DAP 96622 with HyC or MD. To further resolve this effect on AMDase activity by nitrogen, a graph of AMDase activity vs. Ind 18 – 44 h vs. nitrogen was compared (Figure 3.30). AMDase activity increased as Asn concentration increased up to ~ 900 ppm with no apparent relationship above 900 ppm Asn. When runs with MD as the main nitrogen source were compared, the highest AMDase activity was with DAP 96253 and the lowest with DAP 96622. For runs with DAP 96253, there was a highly significant positive correlation between AMDase activity and Asn concentrations < 900 ppm during the t = 18 – 44 h time period. The three runs with DAP 96622 had high Asn addition rates (step additions) that began at 12 h or 24 h. When all Asn induced runs performed in the BF were compared, 80 % of runs with DAP 96622 had AMDase activities of 2 U / mgcdw.

Figure 3.18 All BF AC / AN Induced Biomass Yield vs. Inoculum Mass vs. Supplemental Carbohydrate (DMY)



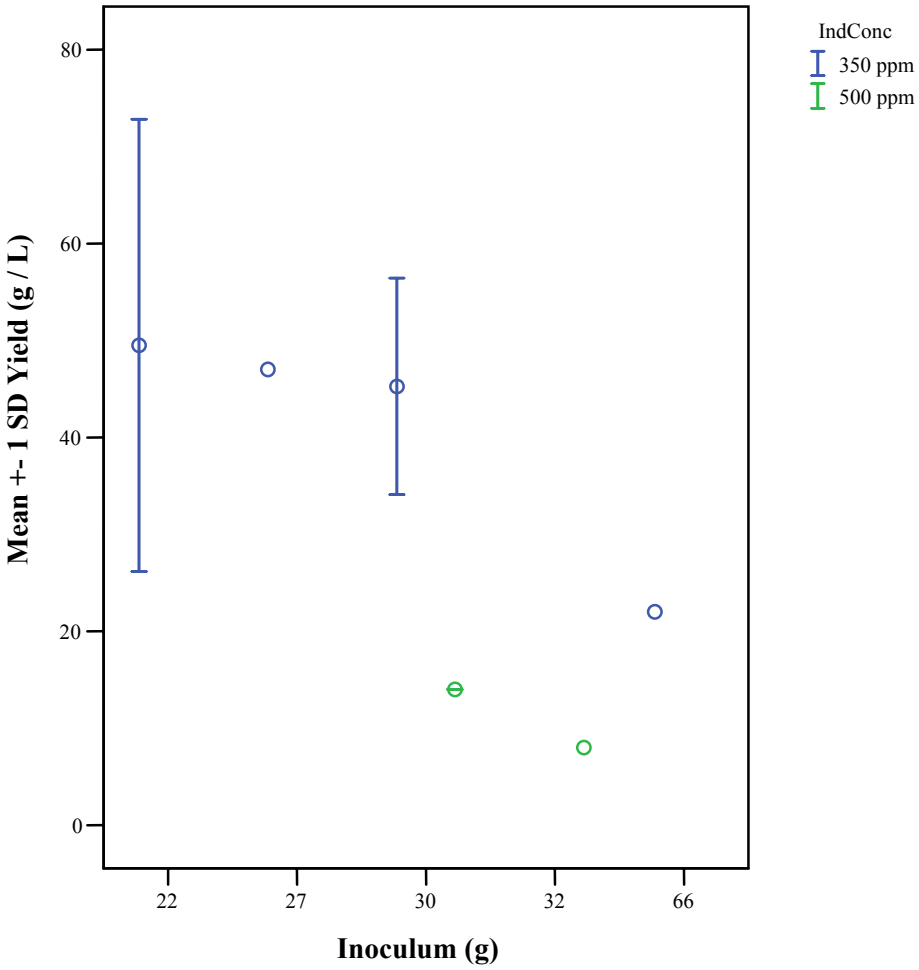
Discrete circles, n = 1; 20 g : none, n = 2; 30 g : none, n = 30 g : dextrose, n = 3
32 g : none, n = 2

Figure 3.19 All BF AC / AN Induced Biomass Yield vs. Inoculum Mass vs. AC / AN Concentration



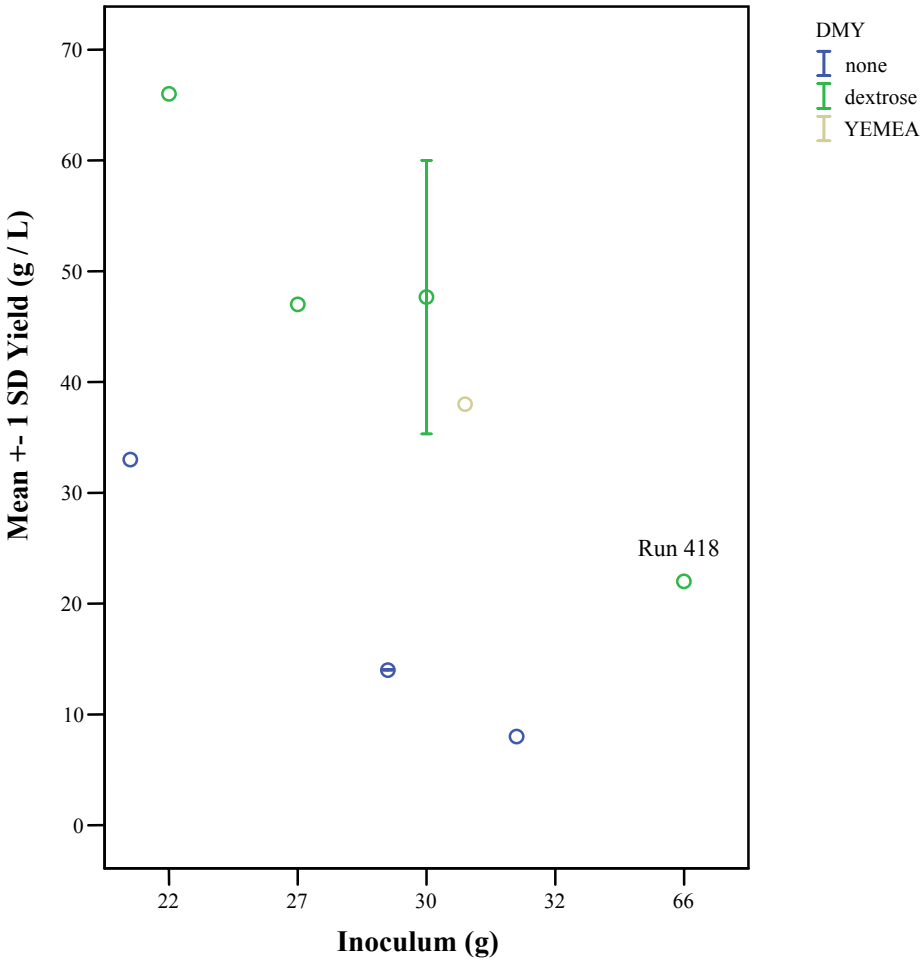
Discrete circles, n = 1; 20 g : 500 ppm, n = 2; 22 g : 350 ppm, n = 2; 30 g : 350 ppm, n = 4; 30 g : 500 ppm, n = 3; 32 g : 500 ppm, n = 2

Figure 3.20 BF Cluster and BF Mixed Cluster AC / AN Induced Biomass Yield vs. Inoculum Mass vs. AC / AN Concentration



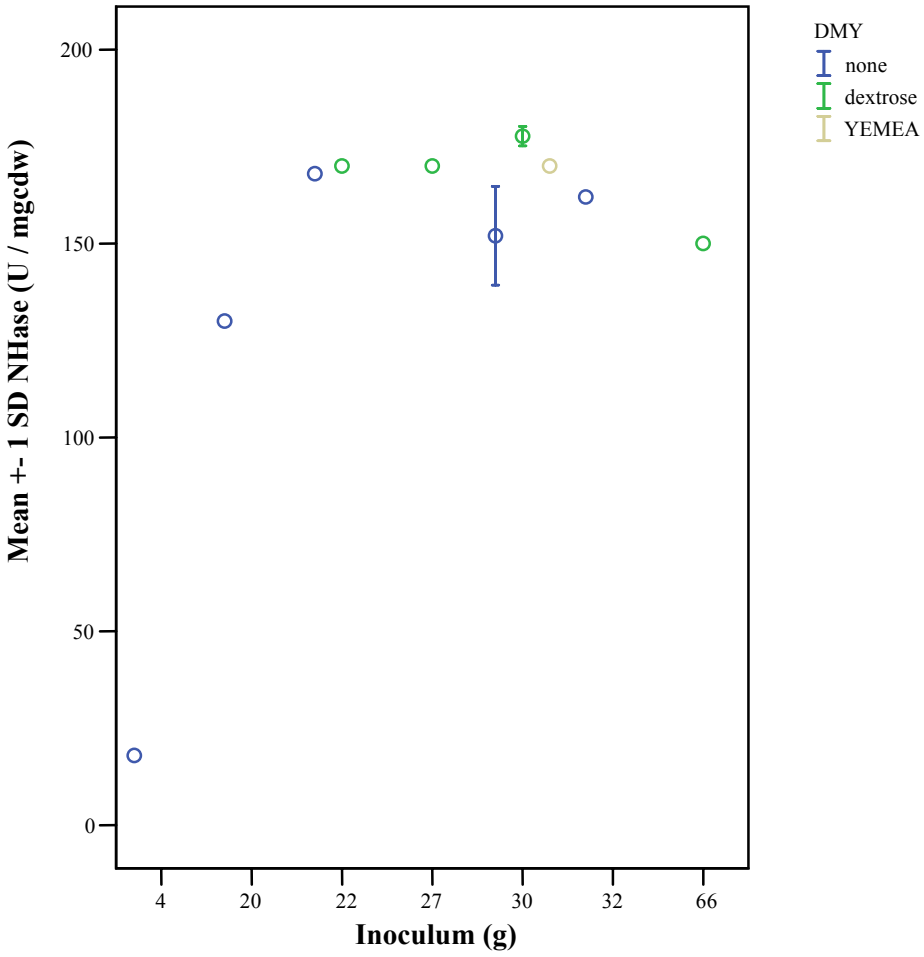
Discrete circles, n = 1; 22 g : 350 ppm, n = 2; 22 g : 350 ppm, n = 2; 30 g : 350 ppm, n = 4

Figure 3.21 BF Cluster and BF Mixed Cluster AC / AN Induced Biomass Yield vs. Inoculum Mass vs. Supplemental Carbohydrate (DMY)



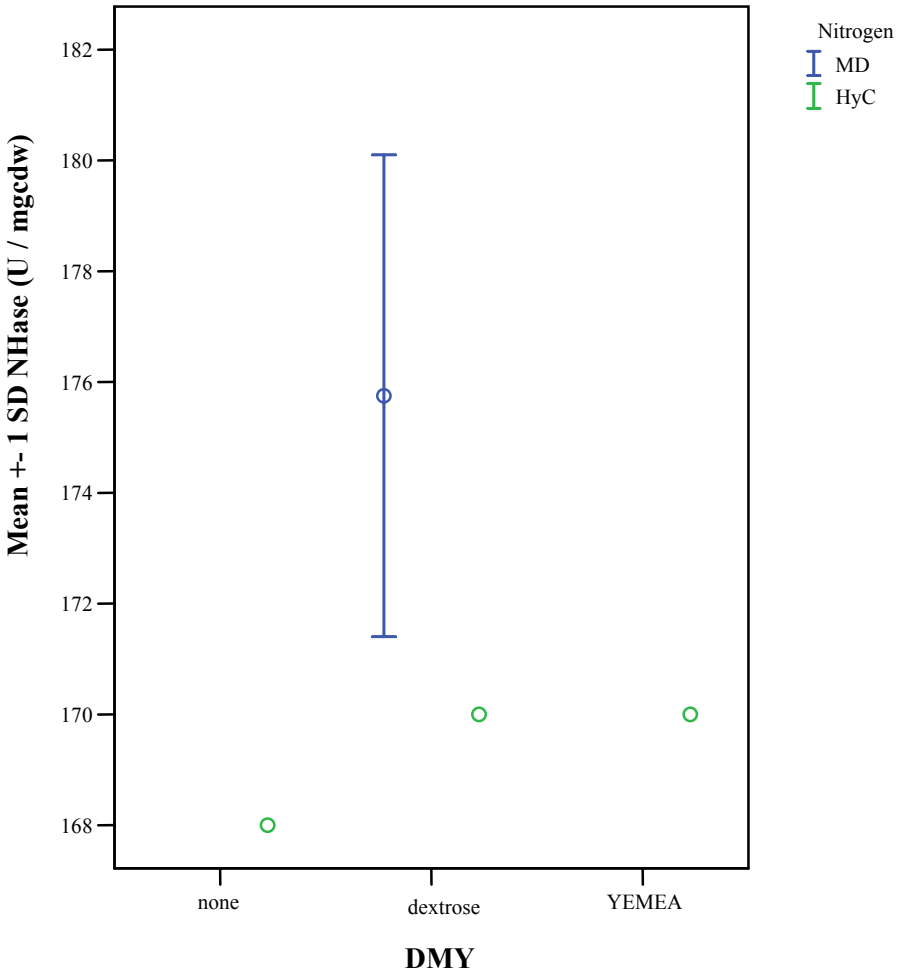
Discrete circles, n = 1; 30 g : none, n = 2; 30 g : dextrose, n = 3

Figure 3.22 All BF AC / AN Induced NHase vs. Inoculum Mass vs. Supplemental Carbohydrate (DMY)



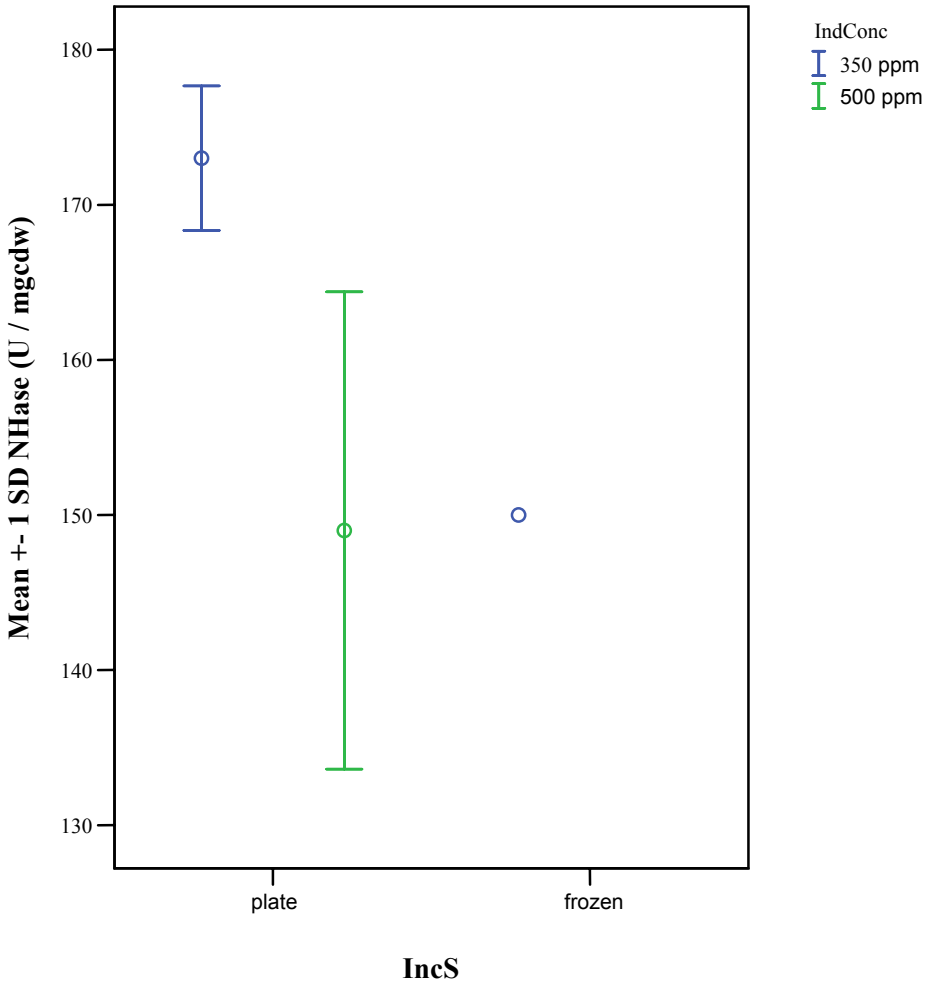
Discrete circles, n = 1; 30 g : none, n = 2; 30 g : dextrose, n = 3

Figure 3.23 BF Cluster AC / AN Induced NHase vs. Supplemental Carbohydrate (DMY) vs. Nitrogen



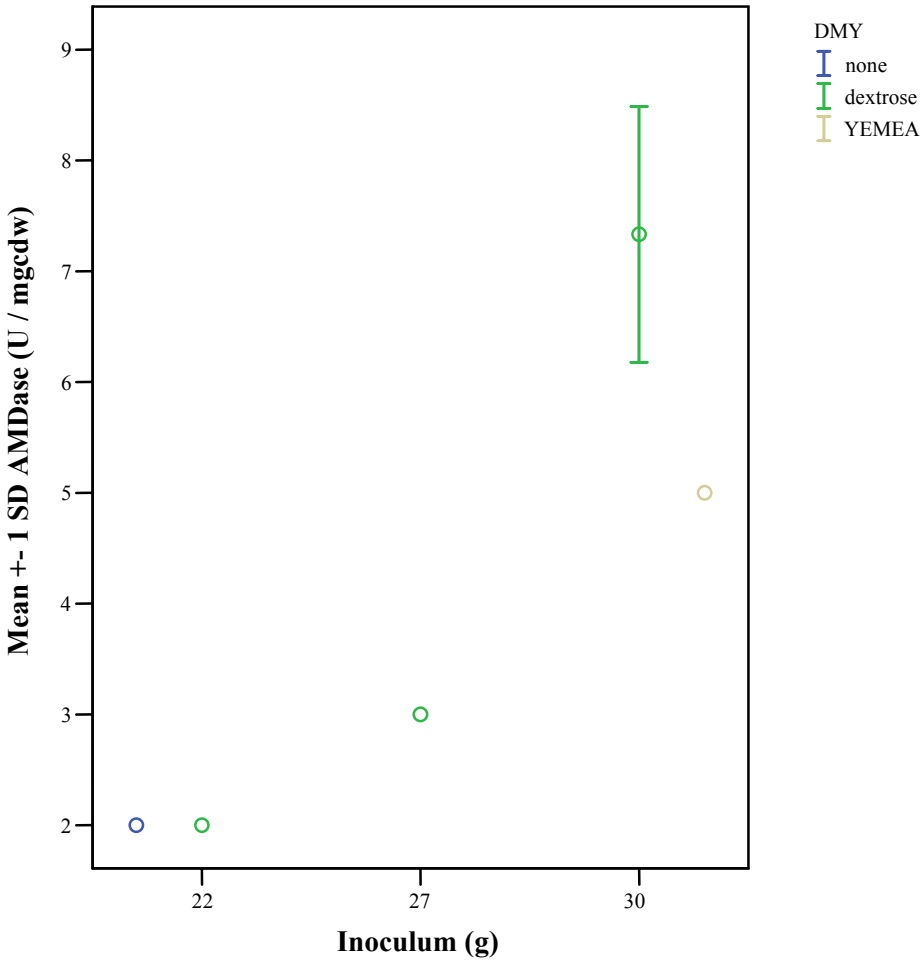
Discrete circles, n = 1; dextrose : MD, n = 4

Figure 3.24 BF Cluster and BF Mixed Cluster AC / AN Induced NHase vs. Inoculum State vs. Inducer Concentration



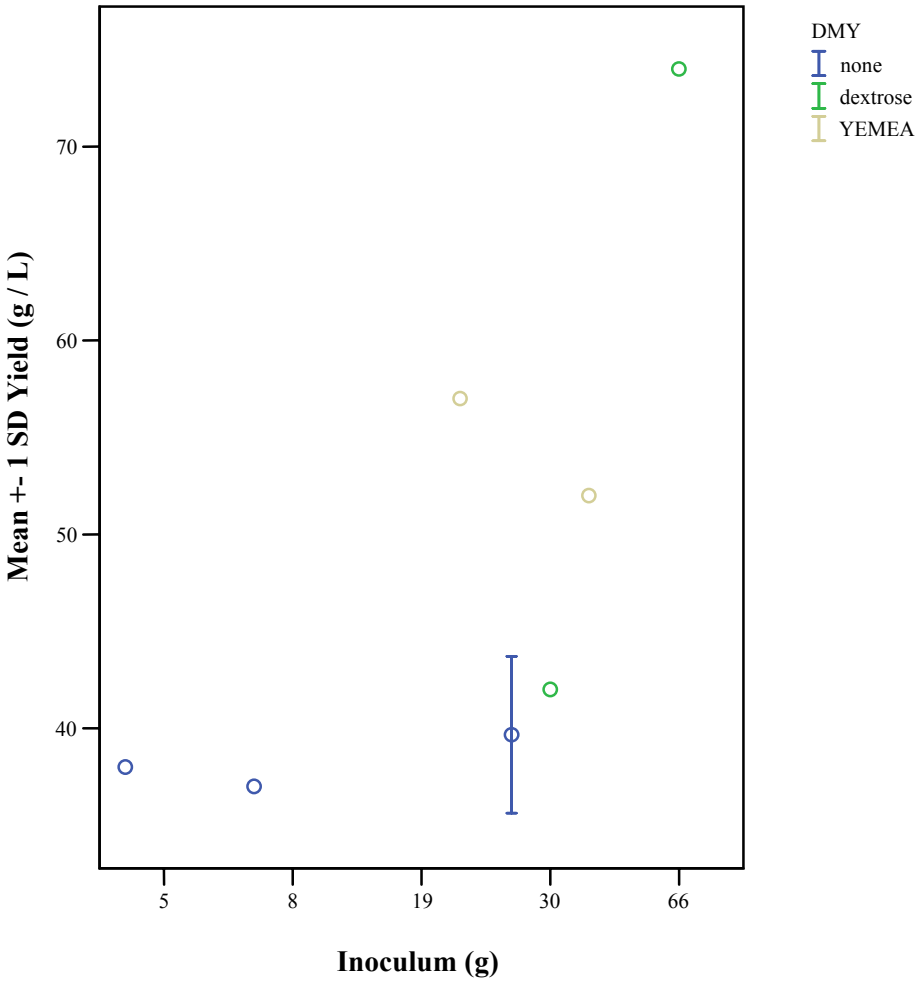
Discrete circles, n = 1; plate : 350 ppm, n = 7; plate : 500 ppm, n = 3

Figure 3.25 BF Cluster AC / AN Induced AMDase vs. Inoculum Mass vs. Supplemental Carbohydrate (DMY)



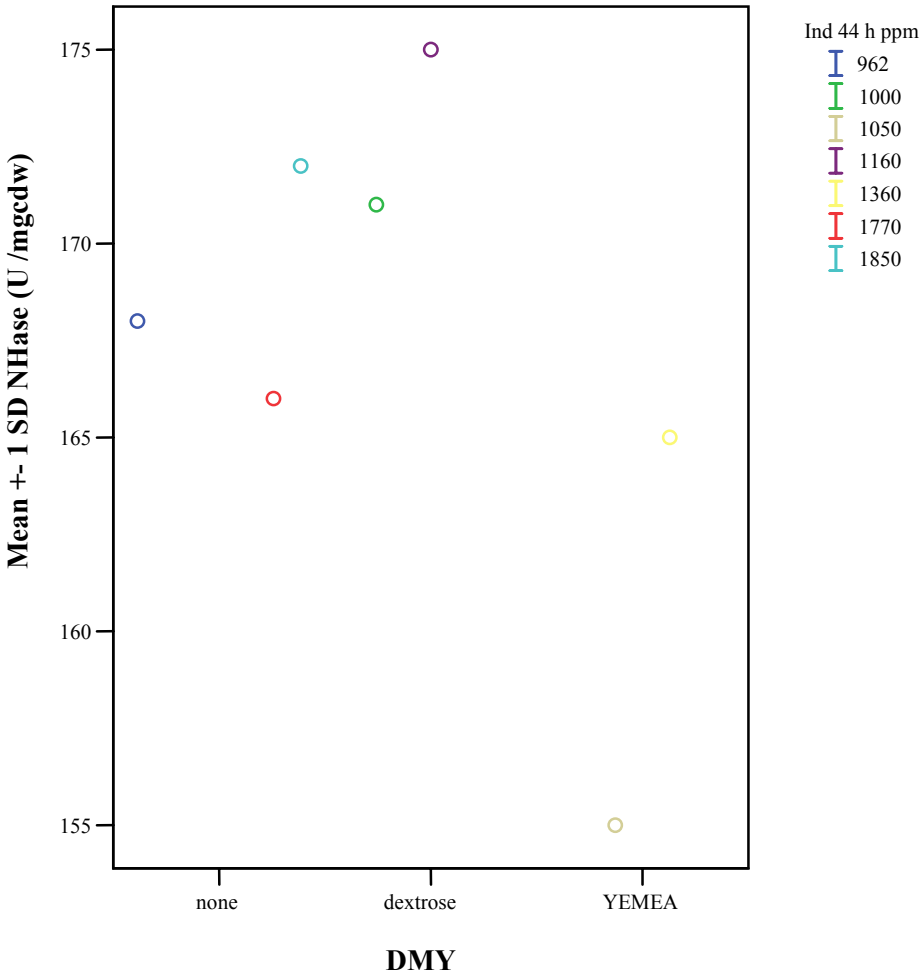
Discrete circles, n = 1; 30 g : dextrose, n = 3

Figure 3.26 BF Cluster and BF Mixed Cluster Asn Induced Yield vs. Inoculum Mass vs. Supplemental Carbohydrate (DMY)



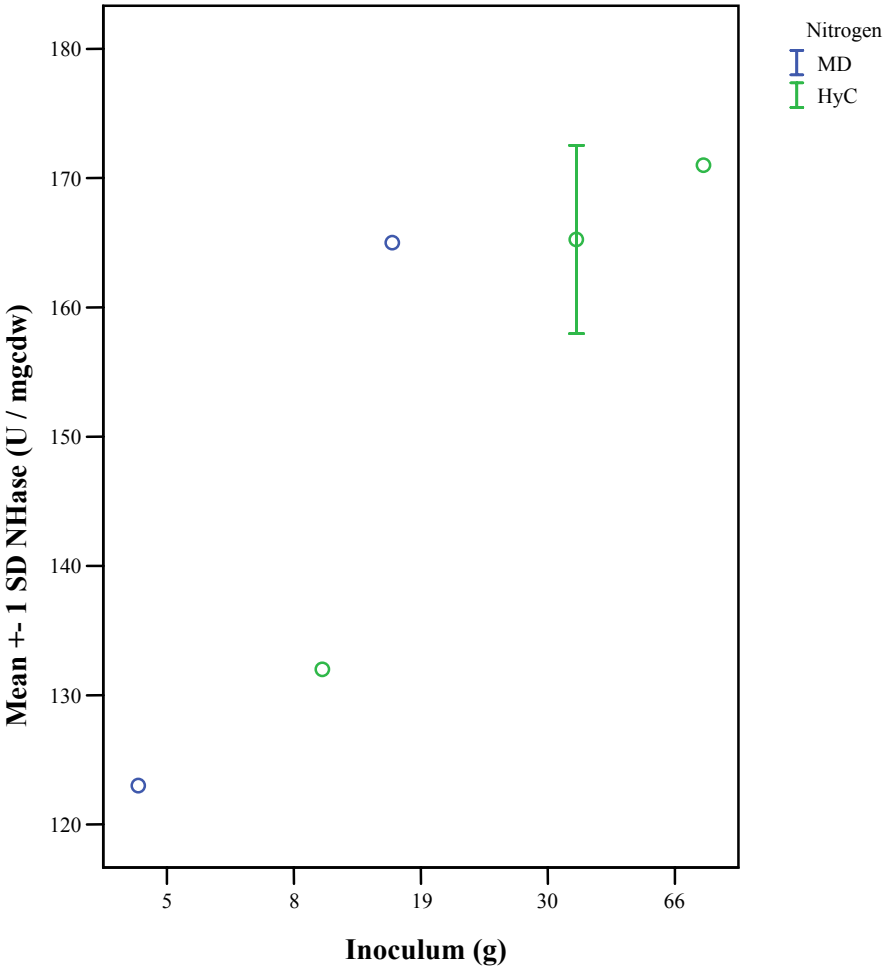
Discrete circles, n = 1; 30 g : none, n = 3

Figure 3.27 BF Cluster Asn Induced NHase vs. Supplemental Carbohydrate vs. Asn ppm 0 – 44 h



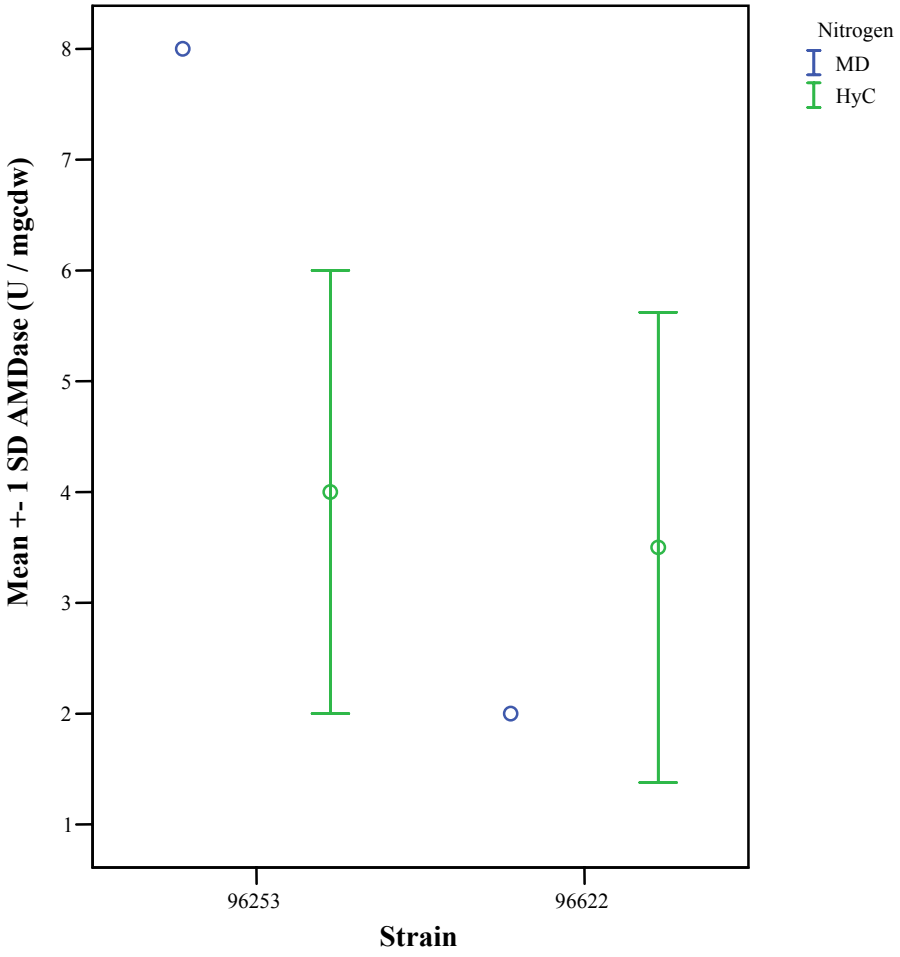
Discrete circles, n = 1

Figure 3.28 All BF Asn Induced NHase vs. Inoculum Mass vs. Nitrogen



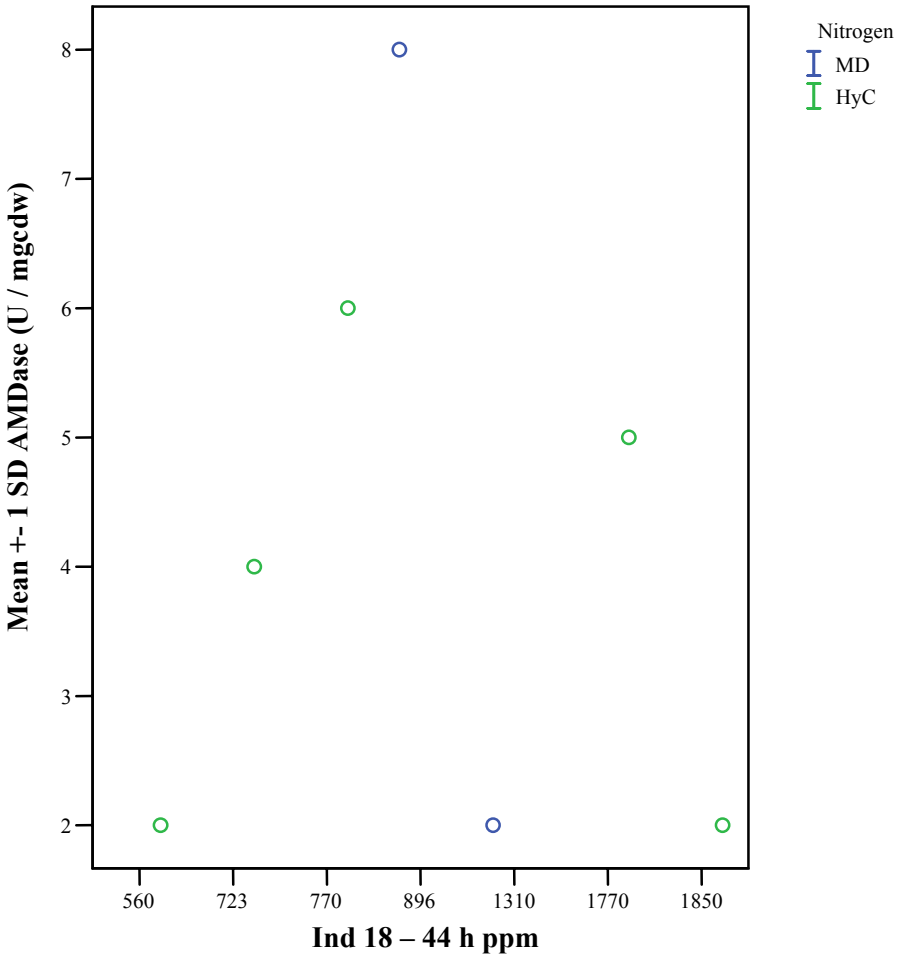
Discrete circles, n = 1; 30 g : HyC, n = 4

Figure 3.29 BF Cluster Asn Induced AMDase vs. Strain vs. Nitrogen



Discrete circles, n = 1; 96253 : HyC, n = 3; 96622 : HyC, n = 2

Figure 3.30 BF Cluster Asn Induced AMDase vs. Asn ppm t = 18 – 44 h vs. Nitrogen



Discrete circles, n = 1

C30 Cluster

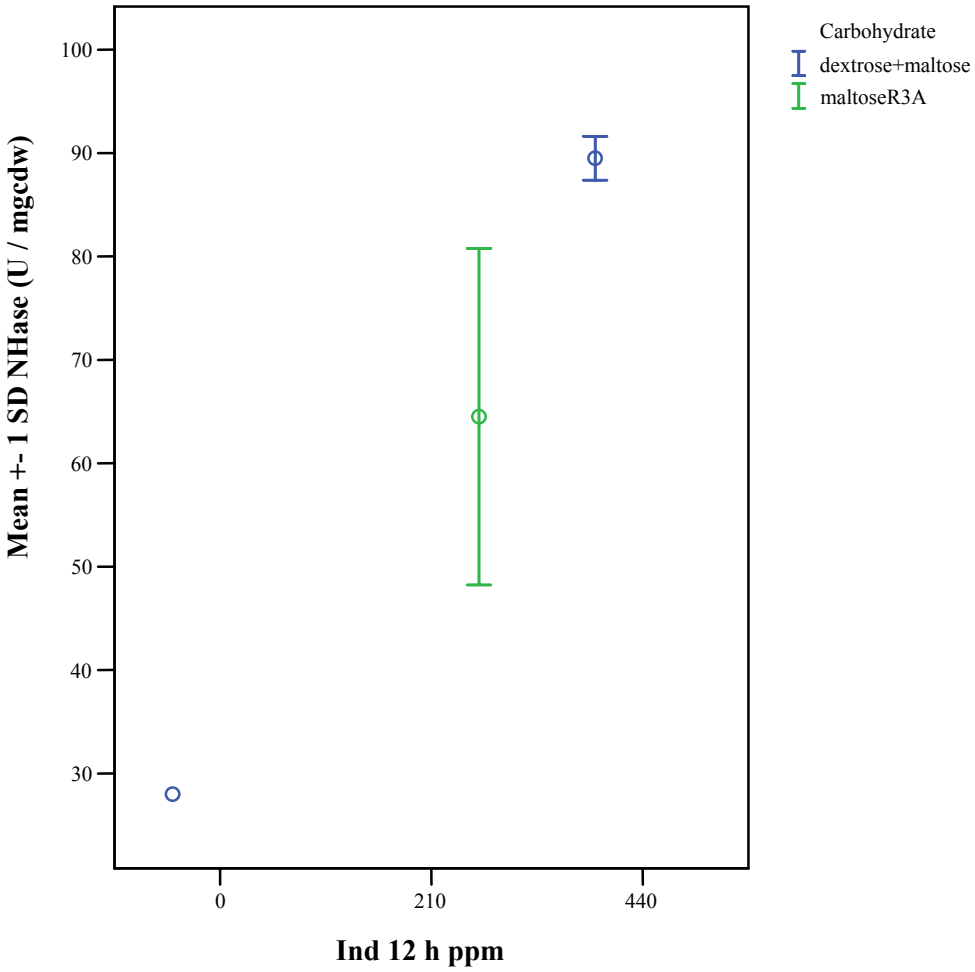
The C30 cluster included runs with low to very high biomass yields (15 – 88 g / L), relatively low NHase activities (< 100 U / mgcdw), and low AMDase activities(≤ 5 U / mgcdw) (Table 3.1 and Figure 3.1). All runs were Asn induced, 89 % of inocula were from biphasic cultures at 15 – 19 g, and 66 % of runs had increased dextrose and / or maltose supplementation. The highest biomass yields were obtained from runs with higher increased dextrose additions (data not shown). Biomass yield also had significant positive correlation with NHase activity. The single low biomass yield (15 g / L cww) was harvested from Run 55 in which GL substituted for dextrose as the major carbohydrate and the biphasic-derived inoculum was 15 – 19 g. Run 53, however, also with GL, produced 52 g / L but had a high (140 g cww) frozen-derived inoculum.

The relationship between carbohydrate and NHase was more complex than that found between carbohydrate and biomass yield. For runs within the C30 Cluster with maltose R3A and R3A (dextrose and maltose) as the major carbohydrate source, there was a highly significant positive correlation between NHase activity and 0 – 12 h Asn concentration (Figure 3.31). Runs with the dextrose equivalent GL and GL + dextrose as the major carbohydrate did not show a trend between NHase induction and 0 – 12 h Asn concentration (NHase Range = 69 – 86 U / mgcdw). Also, as the volumes of dextrose and maltose additions increased, NHase activity increased; with variability by volume and carbohydrate (Figure 3.32). Run 515 with 150 ml maltose obtained higher NHase activity than Run 519 with 80 ml dextrose. However, NHase activities obtained with 700 and 900 ml dextrose in Runs 520 and 521, respectively, were higher than Run 516 with

800 ml maltose addition. When the extra dextrose was 80 ml and initial Asn addition was delayed to 22 h, the NHase activity was the lowest at 28 U / mgcdw. Run 53 with GL as the major carbohydrate source and very high frozen-derived inoculum (140 g) obtained as high NHase activity as runs with high extra dextrose additions.

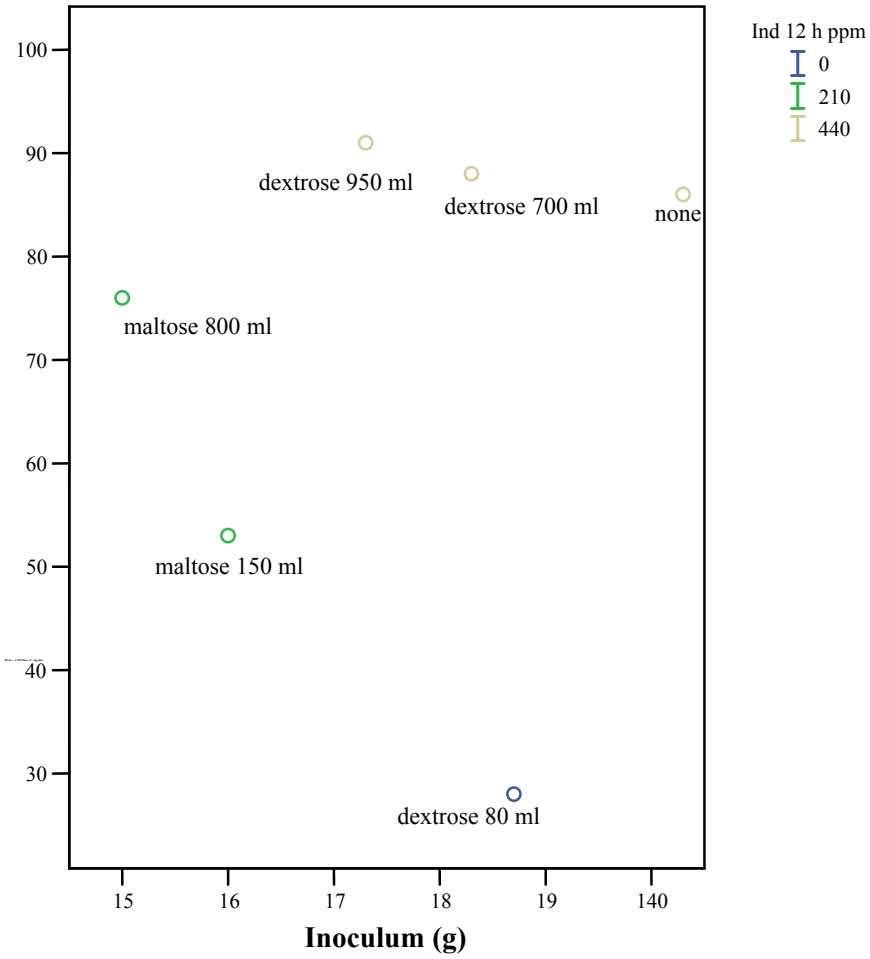
When runs within the C30 cluster that had low (476 µl / min) Asn addition rates that began at 8 h were considered, AMDase activities decreased as inoculum mass increased (Figure 3.33). However, as the inoculum mass was increased for runs with higher (750 – 1000 µl / min) Asn addition rates that were initiated at 8 h, AMDase activities were low and constant. When runs from the C30 Cluster with Asn addition rates = 1000 µl / min were compared, the highest AMDase activity (Run 519, AMDase = 5 U / mgcdw) was induced when the Asn addition start time was delayed to 22 h

Figure 3.31 C30 Cluster Asn Induced NHase vs. 0 – 12 h Asn ppm vs. Carbohydrate



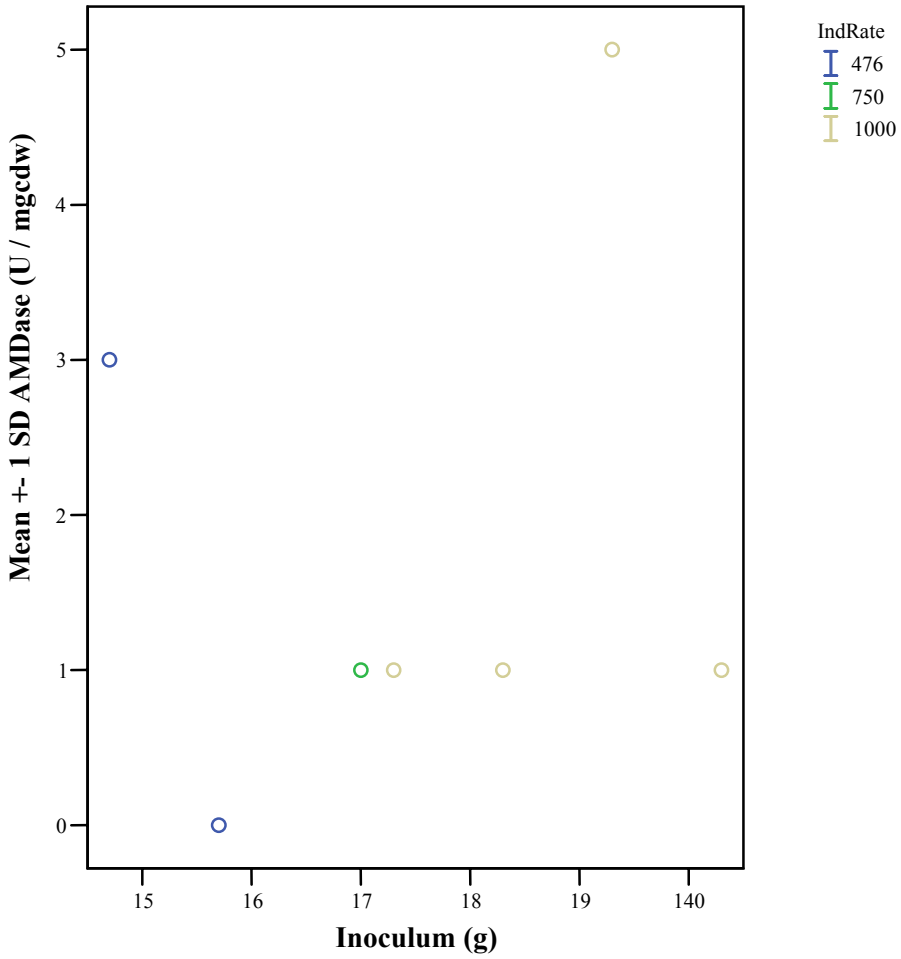
Discrete circles, n = 1; 210 ppm : maltoseR3A, n = 2;
440 ppm : dextrose+maltose, n = 2

Figure 3.32 C30 Cluster Asn Induced NHase vs. Inoculum Mass vs. 0 – 12 h Asn ppm



Discrete circles, n = 1

Figure 3.33 C30 Cluster Asn Induced AMDase vs. Inoculum Mass vs. Inducer Rate



Discrete circles, n = 1

CHAPTER 4 DISCUSSION

Experimental Design

A statistical experimental design was not employed in this study. A conservative, full 2^n factorial approach would have required 512 experiments. While a fractional factorial design would have required far fewer runs, the reduced experiments would have resulted in severely limited versatility. Depending upon which fraction was chosen, it is unlikely that the data obtained would have identified the key parameters that would have enabled feasibility to be achieved. At the time this dissertation research was initiated, the concept of biodetoxification had been clearly established. It was not known, however, if the biodetoxification catalyst production was practical or feasible. The dissertation research was undertaken to provide a framework upon which a fermentation optimization program could be justifiably conducted.

Inducer

Prior to the discovery that asparagine and / or glutamine could induce high levels of AN-specific NHase, all previous work had shown that nitriles and specifically AC and AN were required to induce the high levels of AN-specific NHase used in biodetoxification. Although acetonitrile (AC) and acrylonitrile (AN) are excellent inducers of high-molecular-mass NHase, AC and AN significantly inhibit growth in fed-batch fermentations at total concentrations ≥ 500 ppm. Nitriles are known to inhibit oxidative phosphorylation (Block 2000)[45]. The effects of AC / AN could be reduced by either an increased inoculum mass and / or a delayed initial addition of AC / AN.

Also, reduction of the total AC / AN concentration to ≤ 350 ppm coupled with a slow continuous feed rate greatly reduced inhibitory effects on biomass by AC and AN and preserved high NHase induction.

During the investigation with nitrile and dinitrile inducers, it was decided to include asparagine (Asn) and glutamine (Gln) (both AC and AN are toxic and flammable). It can be seen that both Asn and Gln bear a structural similarity to the metabolites of dinitriles (Figures 4.1 and 4.2). The Asn concentrations used were not inhibitory toward growth or NHase induction, and Asn induced runs achieved the highest biomass yields for both vessels. The highest NHase activity achieved in an Asn induced run was as high as that achieved in reduced concentration AC / AN induced runs (175 vs. 180 U / mgcdw, respectively).

AMDase induction is significant when considering AMD production (BioAMD) from AN. The very lowest levels (< 0.1 U / mgcdw) of or in fact zero AMDase activity is ideal for BioAMD. Early work on NHase genes showed that when NHase was induced, AMDase was actually transcribed first (Ikehata et al. 1989)[21]. Subsequently, Komeda et al. (1996)[30] showed that read-through of the AMDase gene (*amdA*) occurs with L-NHase, but not H-NHase. Therefore, high induction of H-NHase may occur without significant induction of AMDase. Both AC / AN and Asn induced AMDase activity in runs with either strain DAP 96253 or DAP 96622, but DAP 96622 had lower AMDase induction vs. DAP 96253. Although there were a number of runs with AMDase = 1 U / mgcdw, Run 512 was the only run within this investigation that measured AMDase activity = 0 U / mgcdw. This potentially makes whole cells of DAP 96253 and

DAP 96622 less desirable than purified NHase for AMD production. If treatment of nitrile wastewaters is the only concern, then AMDase activity is desirable, and DAP 96253 achieved the highest AMDase activities.

Carbon / Nitrogen

Both carbon and nitrogen sources affected biomass and / or enzyme induction. Proteose Peptone #3, an animal-derived nitrogen source, compared negatively for biomass with comparable runs, and Proflo® (cottonseed) induced high NHase activity but interfered with biomass recovery, resulting in significant losses of cell mass. At the time the time the dissertation research was initiated, highly soluble (low solids) cottonseed protein products were not available. The more soluble cottonseed hydrolysates HyC and MD improved biomass recovery and NHase induction. Nitrogen sources differ as to the percentage of total nitrogen (N), N as amino groups, and free amino acids, as well as suspended solids and the source from which the nitrogen originated. Any of these differences alone or in combination may have contributed to these effects.

Because the rhodococci are slow-growing organisms, the use of glucose alone as the carbohydrate source increased the potential for contamination by glucose utilizing strains. Also, when only glucose was used as the carbohydrate, NHase levels were not that high. The rhodococci are able to utilize maltose by converting it to glucose, so the potential for contamination by glucose utilizing strains was decreased by using a carbohydrate source that contained both glucose and maltose.

Inoculum

The availability of a pre-prepared inoculum would provide consistent results for biomass yield and enzyme activities and reduce the time of inoculum preparation for biphasic (5 – 7 days) and plate-derived (7 – 14 days) inocula. Frozen-derived inocula proved insufficient for achieving high biomass and high NHase activity, unless provided at masses > 3 times that of either plate- or biphasic-derived inocula. Multiple freeze / thaw cycles resulted in loss of viability and loss of NHase activity probably due to ice crystals forming during freezing and again upon thawing.

Low inoculum mass significantly reduced the capacity for biomass production and NHase induction for each inoculum source; plate, biphasic, and frozen. There is a minimum inoculum mass required for high biomass and NHase activity yields. A 48 h run allows limited susceptibility to contamination, and reduces costs associated with heating, cooling, and aeration. Because it is a limited time, slower growing *Rhodococcus* cannot "catch up" if initially below a threshold level.

Scale-up

Although runs in both the BF and C30 were able to achieve high biomass and high NHase activities, there were differences noted in the yields. The highest biomass was achieved in the C30, as was the highest AMDase activity. The highest biomass together with the highest NHase activity was achieved in the BF. The vessels differ in proportional-integral-derivative control (P-I-D) of heating and cooling, mixing, aeration

and dimensions such as height : diameter. Because of these differences, scale-up is often not linear and requires additional experimentation within the ranges set by earlier bench-scale runs to achieve similar results.

Process Improvements

The control of fermentation processes increases with greater use of technology. All parts must function as designed in order to obtain consistent results. During some of these studies, components including the glucose analyzer and inducer pump failed to initiate as programmed. Although SCADA software can provide responses to equipment malfunctions, it cannot, for instance, replace a failed membrane in a biochemical analyzer. Both of these situations adversely affected biomass yield and NHase results. Process improvements, therefore, would include 24 h monitoring of variables via remote communication with off-site computers for more timely interventions.

Additionally, fed-batch fermentations are limited by the working volume of the vessel. Once the solubility limit of a nutrient constituent is reached, the only way to increase the concentration of the desired constituent is by increasing the volume. This in turn may dilute other vital constituents such as the inducer, leading to lower enzyme activities. For runs that utilized maltose or DE as the major carbohydrate source, yields did not reach those obtained when free glucose was used as the main growth substrate; however DE / maltose are less likely to support contaminant growth. Di- and polysaccharides must be hydrolyzed to glucose before being utilized for growth substrate; therefore, a longer fermentation run may be required to allow for more complete

utilization of maltose and DE. Because maltose is a disaccharide of glucose, the potential, however, exists for greater biomass than that obtained with free glucose for an equivalent volume of nutrient.

Improvements in yield would be limited then by the ability to harvest the cells. As the biomass density increases, the ability to pellet cells declines. Current losses of cell mass occur up to ~1 % in foam hold-up in the vessel as the broth is aseptically removed as well as in broth hold-up in the Powerfuge®.

In order to improve NHase activity, a balance would need to be reached with the biomass increase. At 0.2 M, Asn is near the solubility limit at 20 ˚C. In order to avoid the previously mentioned dilution effect, the timing of Asn additions could be further improved for greater and more efficient NHase induction.

Summary

Over 12 variables were evaluated in the effort to elucidate factors that may affect the optimization of fermentation of *Rhodococcus rhodochrous* DAP 96622 and *Rhodococcus* sp. DAP 96253. The factors with the greatest influence were shown to be the inducer, the inducer concentration, inoculum mass and source as well as the major carbohydrate and nitrogen source. In addition, this dissertation is the first to report high NHase induction by Asn in a fed-batch fermentation system. Prior to this program, 250 mg of cells (wet weight) per liter could be provided in 4 – 10 days with an activity of 1 U NHase per mg of cells (dry weight). Current production is > 50 g / L in 48 h with an NHase activity > 150 U / mg of dry cell weight.

Figure 4.1 Induced Nitrile Degradation by DAP 96253 and DAP 96622

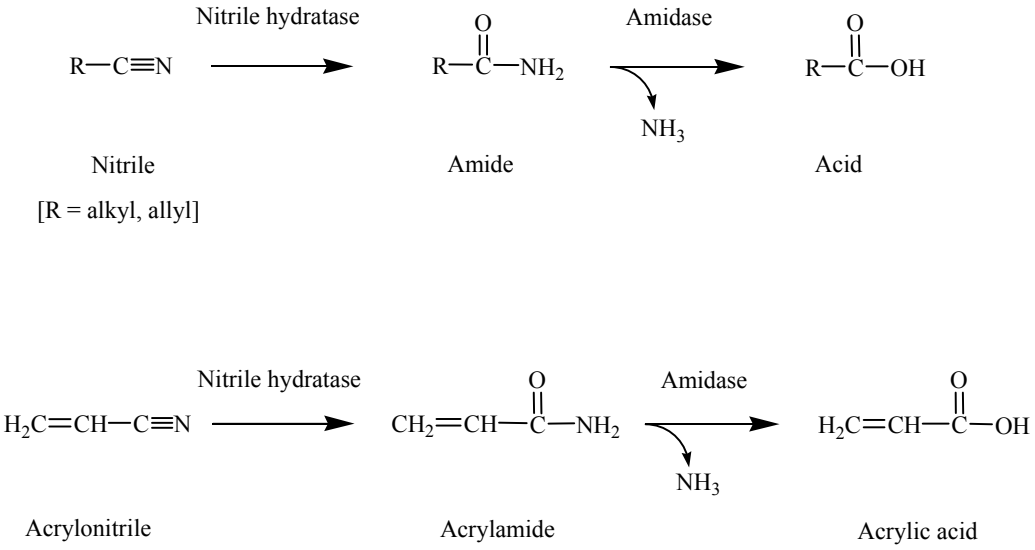
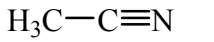
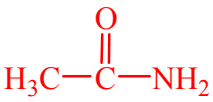


Figure 4.2 NHase Inducer Structures

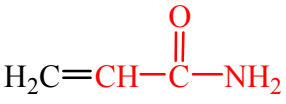


Acetonitrile (AC)

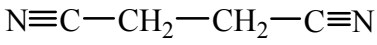
Acrylonitrile (AN)



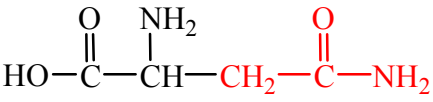
Acetamide



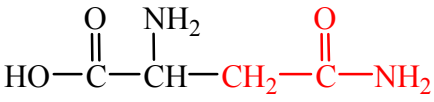
Acrylamide



Succinonitrile



Asparagine (Asn)



Glutamine (Gln)

Amide moieties highlighted in red.

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APPENDIX A

R2A and R3A Media Composition

	Concentration (g / L)	
Component	R2A	R3A
Yeast extract	0.5	1.0
Difco Proteose Peptone #3	0.5	1.0
Casamino acids	0.5	1.0
Glucose	0.5	1.0
Soluble starch	0.5	1.0
Sodium pyruvate	0.3	0.5
K_2HPO_4	0.3	0.6
$MgSO_4 \cdot 7H_2O$	0.05	0.1

Reasoner and Geldreich (1985)[44].

APPENDIX B

YEMEA MEDIA COMPOSITION

Component	Concentration (g / L)
Yeast extract	16.0
Dextrose	26.6
Maltose · H_2O	64.0
Glycerol	10.1
Protein hydrolysate	4.0

Modified from Dietz and Thayer (1980)[12].

APPENDIX C

BioFlo® 3000

APPENDIX D

BioStat® C30